Removal of fluoroquinolones: biosorption in activated sludge and aerobic granular sludge

Vanessa Regina Azevedo Ferreira

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Supervisors:
Professor Doutor Carlos Manuel Magalhães Afonso (FFUP)
Professora Doutora Maria Elizabeth Tiritan (CESPU)
Professora Doutora Paula M. L. Castro (ESB-UCP)

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I dedicate this thesis to my parents.

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Abstract

Antibiotic residues of veterinary and human use have been detected in various environmental matrices, in particular wastewater. Although present in trace levels (μg L^{-1}, ng L^{-1}), these compounds are bioaccumulative, pseudo-persistent and can promote resistance/alterations in bacterial populations. Biosorption is considered to be one of the most promising techniques for wastewater treatment. In recent decades there has been an increasing interest in biosorbents with satisfactory biosorption capacity, low cost and low environmental impact.

This work had as main objective the study of the biosorption process of three fluoroquinolones, ofloxacin (OFL), norfloxacin (NOR) and ciprofloxacin (CPF), using two ecological/green and economical biosorbents: activated sludge (AS) and aerobic granular sludge (AGS). The effect of pH as a promoter of AGS desorption process was also evaluated. The work developed implied the validation of an analytical method, previously developed, for the quantification of OFL, NOR and CPF in aqueous matrices, the characterization of the biosorbents (AS and AGS) and the study of the biosorption kinetics and isotherms in AS and AGS. The effect of pH medium on the AGS desorption process was also evaluated.

AS demonstrated better performance for the removal of OFL, NOR and CPF with a maximum biosorption value of 4.3 mg g_{TSS}^{-1}, 8.2 mg g_{TSS}^{-1} and 11.1 mg g_{TSS}^{-1} respectively, while for AGS the maximum biosorption was 0.77 mg g_{TSS}^{-1}, 0.88 mg g_{TSS}^{-1} and 0.89 mg g_{TSS}^{-1} for CPF and NOR OFL respectively. The biosorption kinetics of both biosorbents indicated that pseudo-second-order equation fitted better to the experimental points. The equilibrium data for AS showed a better fit to the Langmuir model, while the one that better predicts the behavior for AGS was the Freundlich model. The influence of pH on the desorption process in AGS showed a higher desorption of OFL, NOR and CPF at pH 3 and pH 9. At pH 4 the biosorption process was promoted allowing a greater efficiency of AGS.

Keywords: Fluoroquinolones; Biosorption; Desorption; Activated sludge; Aerobic granular sludge

Resumo

Resíduos de antibióticos, de uso humano e veterinário, têm sido detetados em diversas matrizes ambientais, particularmente em águas residuais. Embora presentes em níveis vestigiais (µg L^{-1}, ng L^{-1}), estes compostos são bioacumuláveis, pseudo-persistentes e podem promover resistências/alterações nas populações bacterianas. A biossorção é considerada uma das técnicas mais promissoras para o tratamento de águas residuais. O interesse em materiais biossorventes com grande capacidade de biossorção, de baixo custo e reduzido impacto ambiental tem aumentado nas últimas décadas.

O presente trabalho teve como principal objetivo estudar o processo de adsorção de três fluoroquinolonas, ofloxacina (OFL), norfloxacina (NOR) e ciprofloxacina (CPF) a dois biossorventes ecológicos e económicos (lamas ativadas (AS) e grânulos aeróbios (AGS)). O efeito do pH como promotor do processo de dessorção em AGS também foi avaliado. A investigação realizada envolveu a validação de um método analítico, previamente desenvolvido, para a quantificação de OFL, NOR e CPF, em matrizes aquosas, a caracterização dos materiais biossorventes (AS e AGS), o estudo da cinética e isotérmica de biossorção nas AS e AGS e o efeito do pH do meio, no processo de dessorção em AGS.

As AS demonstraram melhor desempenho na remoção da OFL, NOR e CPF com um valor máximo de biossorção de 4.3 mg g_{TSS}^{-1}, 8.2 mg g_{TSS}^{-1} e 11.1 mg g_{TSS}^{-1} respetivamente, enquanto os AGS apresentavam valores de máximo de biossorção de 0.77 mg g_{TSS}^{-1}, 0.88 mg g_{TSS}^{-1} e 0.89 mg g_{TSS}^{-1}, para a OFL, NOR e CPF respetivamente. Os estudos das cinéticas de biossorção, em ambos os casos, indicaram a equação cinética de pseudo-segunda ordem como a mais adequada aos pontos experimentais. Os dados de equilíbrio para as AS apresentaram um melhor ajuste ao modelo de Langmuir, enquanto nos AGS o modelo que melhor prevê o comportamento dos pontos experimentais é o modelo de Freundlich. O estudo da influência do pH no processo de dessorção em AGS determinou uma maior dessorção da OFL, NOR e CPF, a pH 3 seguido do pH 9. O pH 4 promoveu o processo de adsorção, permitindo aos AGS uma maior eficácia.

Palavras-chave: Fluoroquinolonas; Biossorção; Dessorção; Lamas ativadas; Grânulos aeróbios

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List of abbreviations

AGS – Aerobic granular sludge

AS – Activated sludge

BSA – Bovine serum albumin

CPF – Ciprofloxacin

DL – Detection Limit

DNA – Deoxyribonucleic Acid

DWTP – Drinking Water Treatment Plant

EPS – Extracellular polymeric substances

ESB-UCP – Escola Superior de Biotecnologia da Universidade Católica Portuguesa

FD – Fluorescence Detector

FQ – Fluoroquinolone

HPLC – High Performance Liquid Chromatography

ICH – International Conference on Harmonization

NOR – Norfloxacin

OFL – Ofloxacin

QL – Quantification Limit

r^2 – Coefficient of determination

RSD – Relative Standard Deviation

S/N – Signal noise ratio

TSS – Total suspended solids

VSS – Volatile suspended solids

WWTP – Wastewater Treatment Plant

1. Introduction

1.Introduction

1.1 Antibiotic in the environment

In the past three decades, antibiotics and other pharmaceuticals have been detected in various environmental matrices. The first study which showed the presence of antibiotics in the environment was carried out in 1982 by Watts (1). In the referred study, macrolides, sulphonamides and tetracyclines were found in water samples from different aqueous streams of England at concentrations in the order of μg L^{-1} (1). After that, several studies emerged reporting the presence of significant levels of antibiotics in various environmental matrices such as soil, surface water, domestic sewage, hospital and pharmaceutical industries sewage as well in Wastewater Treatment Plants (WWTP) effluents (2-6). Antibiotics are a broad group of universal and widely used drugs that act as anti-infectious agents, both in human and veterinary medicine. Due to the increased consumption of antibiotics and hence their continuous introduction in the environment, the study of their impact in the environment constitute a subject of major concern. The knowledge on the eventual serious problems of antibiotics in the environment is still recent and thus these pharmaceuticals are considered as emerging pollutants.(7). The extensive use of pharmaceuticals compounds and their continuous introduction into the environmental matrices were responsible for their pseudo-persistence (8). The bioactivity of antibiotics may be observed even when present in small quantities. Currently, the development of the analytical techniques and tools allowed the detection of antibiotics at low concentrations (ng L^{-1}; μg L^{-1}) in various environmental compartments (7).

The complexity of the behavior in terms of presence, origin and destination of antibiotics in the environment is a topic that has generated interest in the scientific community. Once in the environment, antibiotics are subject to mobility, transport and degradation, intervening in these processes phenomena such as, sorption, leaching and chemical and biological processing, for example. Such processes promote the accumulation of these compounds in the soil or its transport to other environmental compartments which can be detrimental to public health (9). Although antibiotics are detected at concentrations in the range of ng L^{-1} to μg L^{-1}, their presence in the environment can promote the disappearance of useful microbial strains, as well as the emergence of antibiotic-resistant microorganisms (10).

Antibiotics are continuously released into the environment by different ways, which are shown in Figure 1.

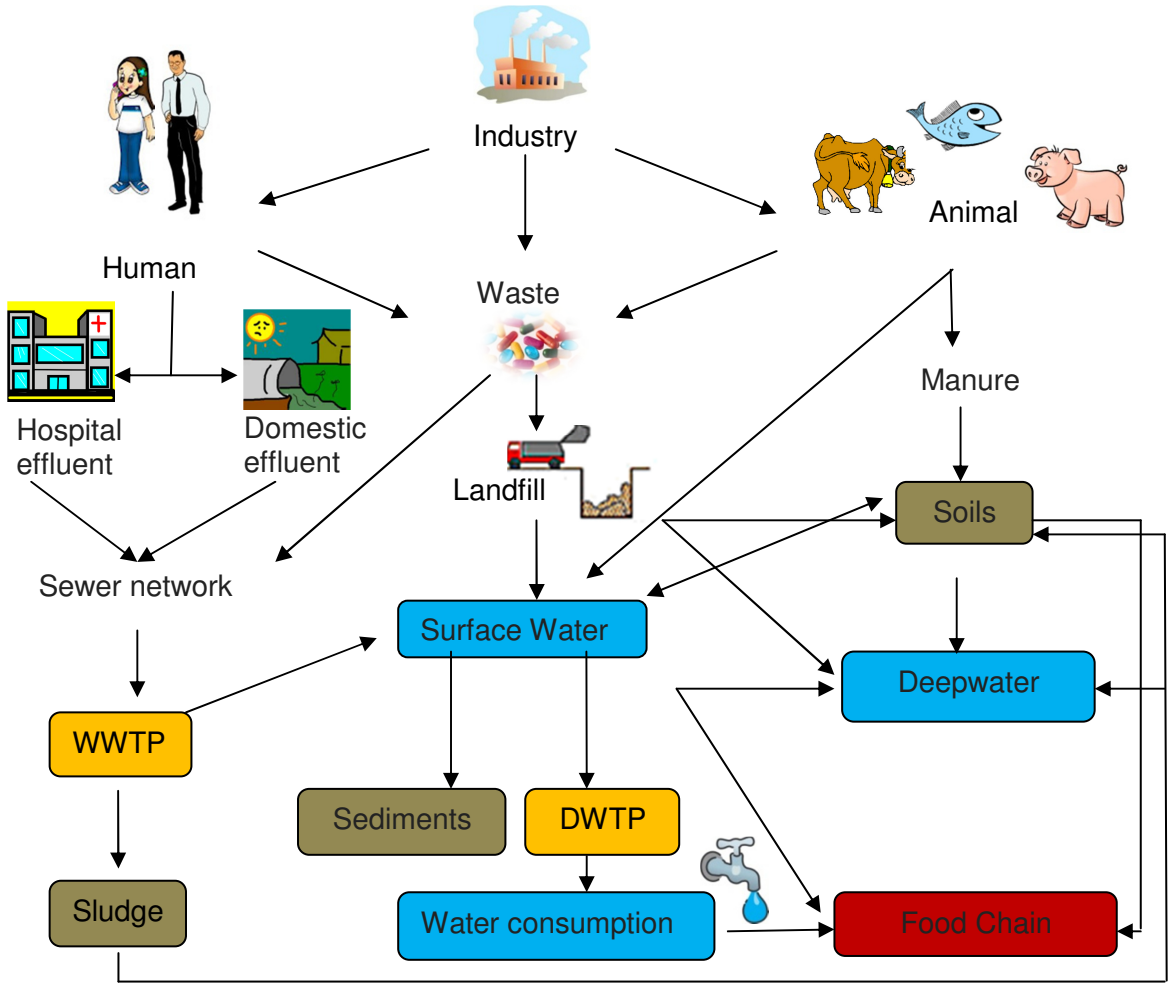


Figure 1. Introduction pathways of **a**ntibiotics in the environment**.**

The antibiotics used either in human or in veterinary medicines are manly introduced in the environment by excretion (11). These compounds could be excreted in its original form or metabolized in accordance with their kind and degree of metabolism (1, 12). The amount excreted depends on the type of substance, the dosage, the age of the user and other factors (1). Another important source of pollution comes from the application of antibiotics in aquaculture (11). The fish farming infections are treated with antibiotics, usually added to the feed and this mixture is directly released into the waters contributing to their accumulation in the surface water and sediments (13). Improper disposal of drugs in the sewage system, such as those that are no longer used, or their deposition in landfills, are also a source of pollution (11). In landfill, the residues of antibiotics may reach surface water and groundwater by leaching. However the WWTP are the most important point of discharge of these micropollutants. Nevertheless, these systems are not

fully prepared to remove these micropollutants and, as such, antibiotics and/or their metabolites eventually end up being released into surface waters (14). Ultimately, surface waters containing these drugs can supply Drinking Water Treatment Plants (DWTP), which also are not equipped to remove residues of these compounds, and thus antibiotics may enter in the drinking water distribution systems (15).

The physical, chemical and biological properties of the antibiotics regulate their fate and behavior in the environment (16).

After antibiotics being released into the waters and soils, processes of sorption (absorption and adsorption), transport (leaching and runoff) and transformation / degradation will be primarily responsible for their fate in the environment. These processes are, in turn, determined by the properties of antibiotics (molecular structure, size, shape, solubility, pka, etc), of the environmental compartments (pH, texture, organic matter, etc) and also the climatic conditions (1).

1.1.1 Fluoroquinolones

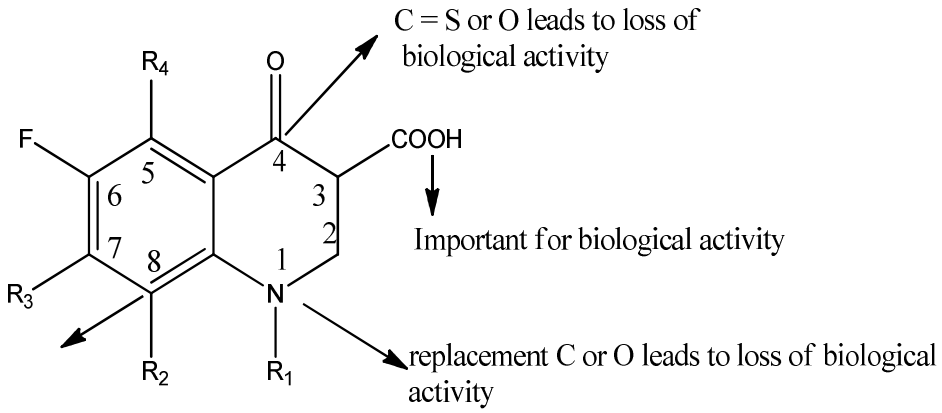
The classic definition of antibiotic is "a compound produced by a microorganism which eradicates or inhibits the growth of other microorganisms, such as bacteria" (9). However, over time this definition has changed and nowadays all natural, synthetic or semisynthetic drugs, which have antibacterial activity, are considered antibiotics.

Antibiotics can be divided into several classes according to various criteria such as, for example, the mechanism of action (17) **:**

- inhibition of the cell wall synthesis (β-lactams);
- inhibition of the replication and transcription of nucleic acids (quinolones e fluoroquinolones);
- inhibition of protein synthesis (tetracyclines, aminoglycosides and macrolides);
- inhibition of the synthesis of essential metabolites (sulphonamides).

Fluoroquinolones (FQs) are synthetic antibiotics with a broad spectrum of action against gram-negative and gram-positive bacteria (17). These antibiotics are a sub-class of quinolones. The first quinolone, the nalidixic acid, was discovered in 1962 by George Lesher (18). The FQs emerged later in the 80s by a structural modification of quinolones (17). The structural changes allowed the creation of compounds with broad spectrum of

action, better pharmacokinetics, lower toxicity and capable of promoting lower levels of resistance on the microorganisms. FQs have in common a bicyclic system, the core 4-oxo-1 ,4-dihydroquinoline, with a carboxylic acid group at position 3 of the pyridone ring and a fluorine atom in position 6 (19) (Fig. 2).



Groups that confer biological activity

R_1 - cyclopropyl; tert-butyl; ethyl; *m*- fluorobenzene; 2,4 - difluorobenzene; OCH_3; NCH_3
R_2 - H; Cl; F; OM_e
R_3 - Piperazine; pyrrolidine
R_4 - F; NH_2

Figure 2. Structure and some structure-activity relationships in FQs (adapted (19)).

Currently, there is a variety of FQs available in the market. The FQs act by inhibiting the transcription and replication of bacterial DNA (20). In gram-positive bacteria, they stabilize the complex formed between DNA and topoisomerase IV. In gram-negative bacteria, they stabilize the complex formed between DNA and topoisomerase II, also called DNA gyrase. The stabilization of the enzyme-DNA complex, the FQs prevent the normal processing of replication and transcription of bacterial DNA since they block the separation of the DNA strands, which are essential for bacterial survival and growth (21).

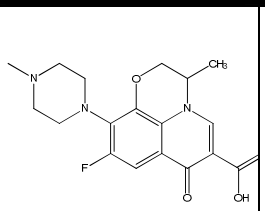
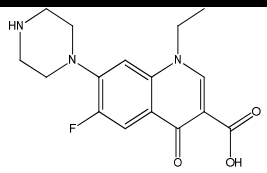
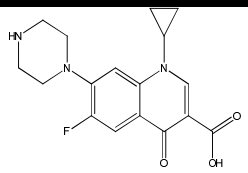
FQs are polar molecules which possess different functional groups in its structure such as carboxyl, carbonyl and amine. These fluorinated antibiotics are reported as having good biosorption capacity in soils (20). The fluorine atom at the C6-position of the basic quinolone nucleus confers unique structural and atomic properties such as a high ionization potential and a low polarity. In terms of size the fluorine atom is similar to hydrogen atom (the smallest of the periodic table) , however it is the most electronegative element of the periodic table (19). As such, the C-F bonds are very strong, with strongly

polarized connections since fluorine has a strong negative inductive effect (22). Thus, the presence of fluorine in a molecule causes physico-chemical properties of fluorinated molecules considerably different from the equivalent hydrocarbons. Moreover, the possibility of replacing a hydrogen atom by a fluorine does not affect the size of the molecule, since both are similar in size, but produces a strong and remarkable effect on the electronegativity (19). These facts are of great importance for the development of new drugs, where, for example, the introduction of a fluorine atom allows to obtain the same electronegativity effect of a hydroxyl group, without having to increase the overall size of the molecule, avoiding steric hindrance that can be decisive for the biological activity (22). On the other hand, the presence of C-F bond, which is the strongest bond carbon can establish with another atom, can change the metabolism of a drug, allowing the modulation of its pharmacokinetics. Likewise, changes in the physicochemical properties caused by the presence of fluorine in a molecule also influence their behavior in the environment. Thus, mobility and degradation of fluorinated molecules are reported to be very different from those of equivalent hydrocarbons (19). The presence of fluorine modify properties of the compounds, such as volatility, surface tension, polarity, solubility, lipophilicity and biosorption (23). The presence of a fluorine atom in a molecule influences its physicochemical properties and has consequences both in terms of biological activity and environmental fate/behavior.

In this work we studied three 2nd generation FQs namely, OFL, NOR and CPF, which are amongst the most widely commercialized antibiotics worldwide (24).

Table 1 shows some physicochemical properties of OFL, NOR and CPF which are important to understand the environmental behavior of these drugs.

Table 1. Physico-chemical properties of OFL, NOR and CPF (adapted (25))

	Ofloxacin	Norfloxacin	Ciprofloxacin
Structure			
Weight	361,37	319,33	331,34
Water solubility (g L^{-1})	1.44	1.01	1.35
Log P	-0.39	-1.03	0.28
Log S	-2.4	-2.5	-2.4
pka1	5.45	5.77	5.76
pka2	6.2	8.68	8.68
Hydrogen acceptors	7	6	6
Hydrogen donors	1	2	2
Polar surface area	73.32	72.83	72.88
Rotatable bonds	2	3	3
Refractivity	94.94	85.43	87.94
Polarizability	36.69	32.26	33.12
Number of rings	4	3	4
Hidrosolubility (g L^{-1})	1.44	1.01	1.35

OFL, NOR and CPF have in common a piperazinyl group at position 7, and the fluorine atom at position 6, which potentiate the effect against gram-positive bacteria and increase their spectrum of activity (26). Thus, the structural differences between the three studied FQs occurs at position 1, since CPF has a cyclopropyl group, NOR an ethyl group and OFL a isobutoxy group.

The presence of an acidic group (carboxyl group) and basic group (amines) confers amphoteric properties to these compounds and thus the species present in solution depends on the pH (20). This is due to the protonation/deprotonation of these groups at different pH values, thus influencing the environmental behavior of these compounds. The proteolytic balance of the FQs in acid, neutral and alkaline media is shown in Figure 3.

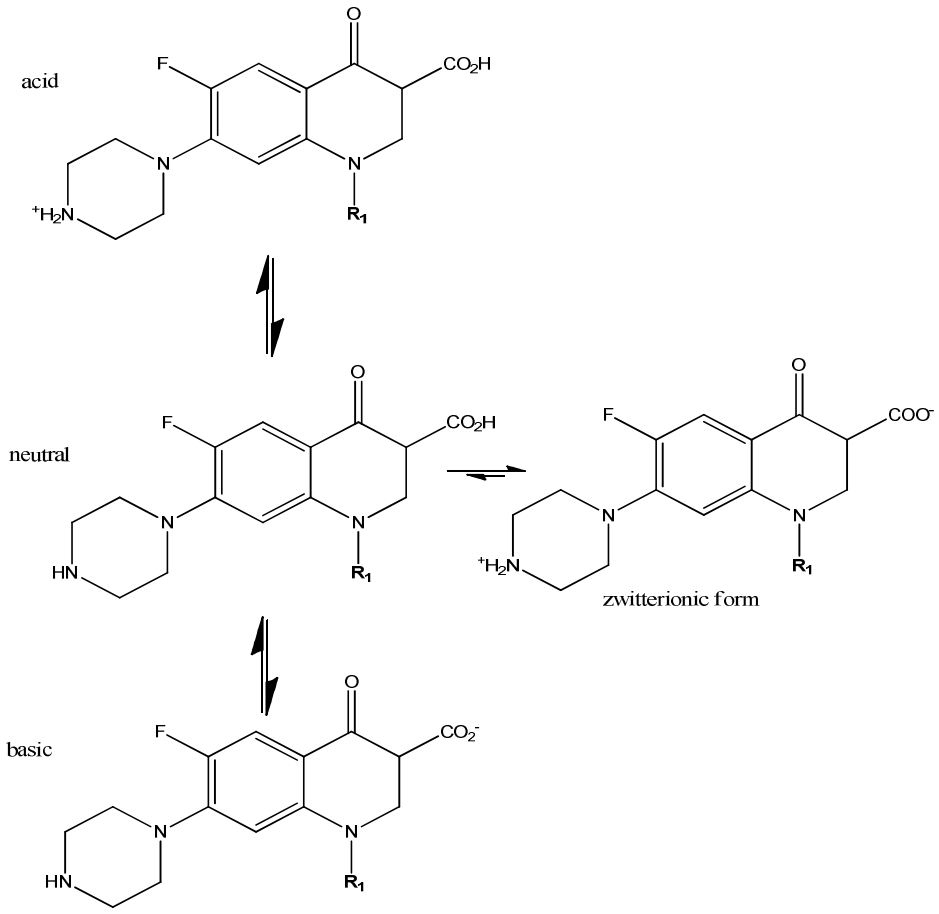


Figure 3. Scheme of protonation and deprotonation of FQs.

Depending on the pH of the medium, OFL, NOR and CPF may exist in the cationic form in acid medium or in the anionic form (in alkaline medium). At pH 7 the FQs are partially ionized, but the predominant species is the zwitterionic form, that coexists with the anionic and the cationic forms (20, 27).

1.1.1.1 Occurrence of OFL, NOR and CPF in environment

In recent decades, antibiotics have been detected in various environmental matrices. Table 2 summarizes some of the studies where the occurrence of FQs in the environment is reported.

Table 2. Occurrence of FQs in various environmental matrices.

Matrix	Source		Antibiotics concentration (ng L^{-1}) (*µg Kg^{-1})			Reference
			CPF	NOR	OFL	
Surface water	River water	Australia	28	28	28	(28)
		Pearl River (Hong Kong)		10-250	20-110	(29)
		Seine River (France)		20-50	30	(30)
		Queensland (Australia)	1300	1150		(31)
		Atabaia River (São Paulo, Brazil)	119	119		(32)
Water consumption	DWTP effluent	China			7	(33)
	Tap water	Macau	2-8	7-17		(34)
		Guangzhou (China)	6-180	Até 83		
Seawater		Victoria Harbour (Hong Kong)		20-30	8-10	(29)
		Victoria Harbour (Hong Kong)		8-27	8-150	(35)
Wastewater	Hospital sewage	Kalmar (Sweden)	3600-101000		200-7600	(36)
		Albuquerque, Santa Fé (New México, EUA)	850-2000		1300-35500	(2)
		Coimbra (Portugal)	127-11000	30-334	353-10700	(37)
		Queensland (Australia)	15000	200		(31)
		Kristiansand (Sweden)	3300-9300	Até 22	58-840	(4)
		China	10-220	140-1620	2340-4240	(33)
		Korea	460-5030			(38)
	WWTP influent	Austria	340	355	1875	(28)
		Portales, Socorro (New México, EUA)	200-1000		110-1000	(2)
		Coimbra (Portugal)	419-667	191-455		(37)
		Queensland (Australia)	1100	220		(31)
		China	458	859	780	(33)
		Varese (Italy)	513		463	(5)
		Korea	124-246			(38)
	WWTP effluent	Brisbane (Australia)	130	210		(39)
		Áustria	395	340	240	(28)
		New Iorque (EUA)	220-450			(40)
		Coimbra (Portugal)	101-309	35-296		(37)
		Hong Kong		6-3700	100-7870	(35)
		Queensland (Australia		250		(31)
		Kristianstad (Sweden)	28-320	Up to27	9-30	(4)
		Varese (Italy)	148		191	(5)
		Korea	61-2050			(38)
	Pharmaceutical wastewater	Korea	528-34600			(38)
		India (Patancheru)	28 000000 - 31 000000	390000 - 420000	150000 - 160000	(41)

Matrix	Source		Antibiotics concentration (ng L^{-1}) (*µg Kg^{-1})			Reference
			CPF	NOR	OFL	
Wastewater	Piggery effluent	China			8	(33)
	Abattoir effluent	China	11	28	24	
Solids	River sediments	Austria			58*	(28)
	WWTP sludge	Austria	230*	150*	510*	
		Varese (Italy)	2*		3*	(5)
Food	Milk (Cow)	South Korea	17*			(42)
	Carrots	Germany		3-5*		(43)

According to the data presented in Table 2, FQs were detected at high levels in effluents from hospitals (10-101000 ng L^{-1}) and effluents from industries producing antibiotics (528 – 34600 ng L^{-1}). Regarding effluents from pharmaceutical companies , it has been reported in the literature that in the countries of Europe and North America they have an insignificant impact (41). However, in Asiatic countries the concentrations of various antibiotics in industrial effluents can reach mg L^{-1} (11).

The study of Seifrtrova (2008) showed significant levels of three the FQS in study in WWTP and hospital effluent in Coimbra (Portugal). Other studies have also detected high concentrations of OFL, NOR and CPF (6-7860 ng L^{-1}) in WWTP, which indicates that the treatment processes are not effective in removing these compounds and, thus, they eventually can reach aquatic compartments, namely surface waters, where considerable levels of FQs (20 - 1300 ng L^{-1}) have been observed. OFL, NOR and CPF were even detected in water consumption (2-180 ng L^{-1}). Despite being present at reduced levels this illustrates the ineffectiveness of the methods used in WWTP and in DWTP.

Detection of FQs in some food is also a subject of great concern due to the possible adverse effects on human health, including allergic or toxic reactions (especially in children) and the contribution to resistance to pathogens (7).From the three FQs studied in this work, CPF is usually present at the highest levels in all different environmental compartments (Table 3). According to the European Centre 2010, the consumption of quinolones in Portugal was 13.3% of all prescribed antimicrobials. CPF accounts for most of the consumption of antibacterial quinolones (24).

1.2 Environmental effects and public health

With the continuous use of antibiotics, these compounds become pseudo-persistent and can promote the development of antimicrobial-resistance in bacteria (7). Resistance is the term employed to designate the ability of bacteria to adjust to changes in the environment and survive (10).

Resistance mechanisms developed by bacteria to the antibacterial action of FQs can occur by two different ways (44)

1. Changes in FQs target molecules: the DNA gyrase and topoisomerase IV enzymes;

2. Changes in the intracellular accumulation of FQs.

A reduced intracellular accumulation may be due to two main factors: the decrease in permeability of the outer membrane to the drug because of membrane proteins (porins) undergo changes that affect its expression and the stimulation of cellular efflux systems that allow to expel the drug from inside the cells by active transport (45).

The appearance of resistance is a vastly complex process and that causes risk to public health. The transference of these resistant bacteria to humans are due to the ingestion of contaminated water or food – if plants are watered with surface water or sewage sludge; if manure is used as fertilizer or even if the resistant bacteria are present in the aliment itself, as the meat (7-10).

In these situations, the ingested concentration, exposure time, and the half-life of the compound, are factors that must be considered (1). Some studies indicate an increased sensitivity to light caused by the ingestion of water or food contaminated with FQs (9). Furthermore bacterial resistance can cause difficulties in the treatment of certain diseases such as allergic or toxic reactions, especially in children, who are more vulnerable thereby promoting the difficulty in controlling diseases, in the development of other chronic diseases and, it also makes more expensive the medical treatments (46).

1.3 Removal in WWTP

The WWTP are specialized infrastructures for the treatment of wastewater influents from different sources. These infrastructures are responsible for treating wastewater in order to

be released back into the environment with lower and acceptable level of pollution in accordance with the legislation in force.

In WWTP the treatment is normally divided in four phases, namely: preliminary, primary, secondary and tertiary treatment (Fig. 4). Some WWTP facilities only have two or three of these stages since the processes of wastewater treatment should be selected according to the specific characteristics of the wastewater to be treated (47).

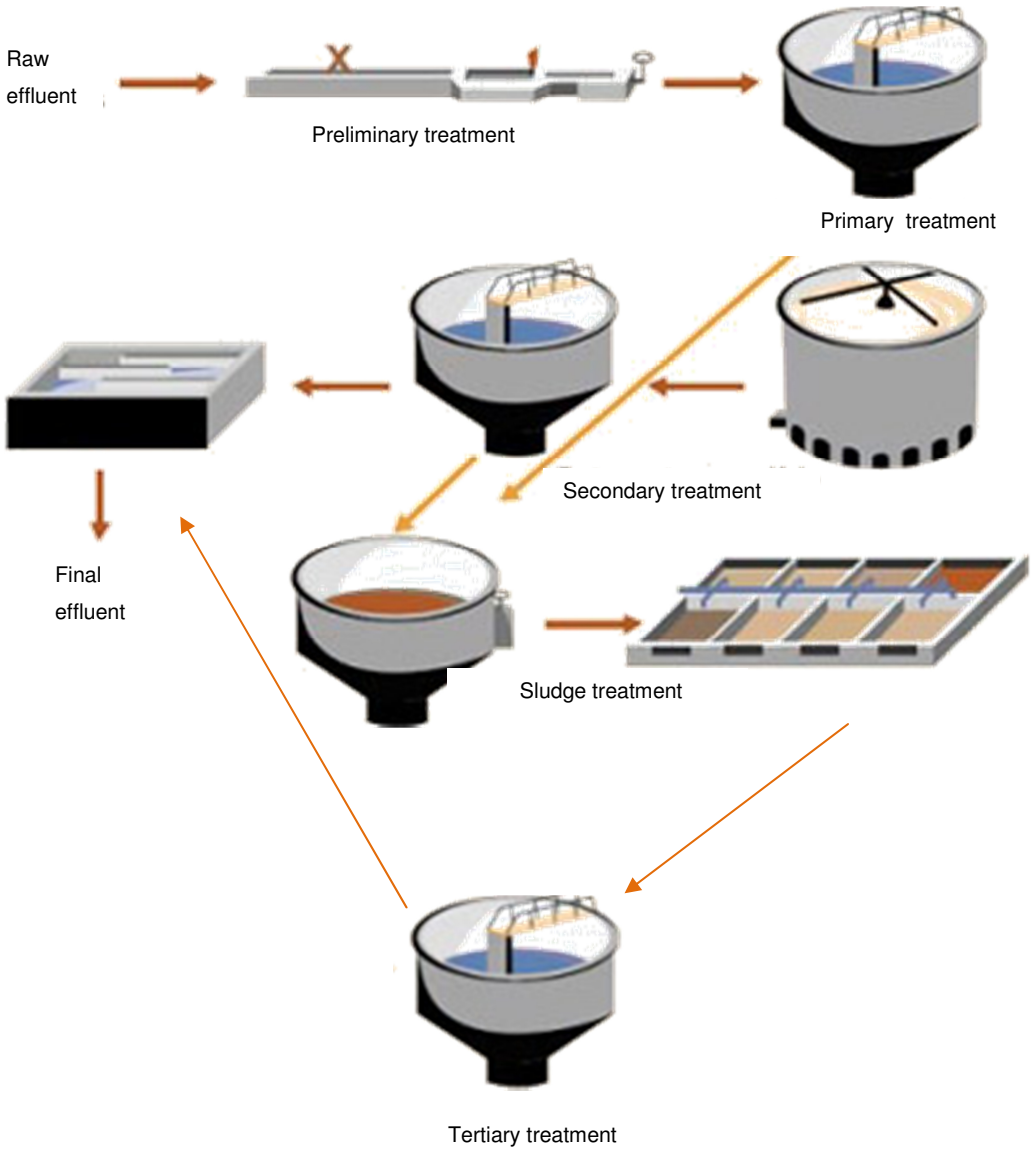


Figure 4. Different stages of a system for wastewater treatment (adapted (47)).

The first step is the preliminary treatment where the grease and sand are removed from the wastewater influent. In the primary treatment, the solid material is separated from the raw sewage by sedimentation. Usually, in this stage the separation occurs exclusively

through physical action but, in some cases, chemicals are often used to accelerate the sedimentation process (47). Secondary treatment uses biological processes in which microorganisms remove the dissolved organic matter in the remaining wastewater. Currently, there are two general types of biological treatments technologies available: suspended biomass (e.g. activated sludge (AS)) and the fixed/attached biomass (e.g. biodiscs) (47). The tertiary treatment is the final stage in WWTP before the discharge of the effluent where a disinfection of the treated wastewater is performed in order to eliminate any pathogen or, in special cases, to remove additional nutrients such as nitrogen and phosphorus (47).

The detection of micropollutants, including pharmaceuticals, in the effluents from WWTP demonstrates the inefficacy of such systems to remove these kind of compounds (29, 31, 32, 38). Thus, such micropollutants are introduced and tend to accumulate and persist in the environment. The concern about their adverse effects on humans and animals tends to increase and the interest in new and better removal methods has risen significantly. Physical and chemical removal processes, such as advanced oxidation, incineration, chlorination, ozonation and combustion require expensive equipment and often generate toxic by-products (48). Therefore, development of biological processes for the removal of aqueous waste containing toxic compounds has received increasing interest. In addition, biological removal techniques have low cost, high efficiency and environmental benefits. In general, the biological treatment of toxic effluents is preferred over chemical and physical methods in terms of efficiency and economy (48).

The biological wastewater treatment using aerobic granular sludge (AGS) is a recent and very promising technology. The treatment system with AGS is simple and compact, since all the processing steps may be performed in a single tank, making it more economic in relation to classic WWTP systems, where a large area surface is required to implement the various treatment units for subsequent separation of biomass (49).

Moreover, AGS have several advantages over the AS, such as:

(i) strong and compact microbial structure;

(ii) high biomass retention;

(iii) excellent sedimentation properties;

(iv) tolerance to chemical toxicity;

(v) high biosorption capacity.

These outstanding features make the AGS much more advantageous over conventional AS systems and other biofilm technologies (50). This technology has been successfully applied for the treatment of different kinds of wastewater including domestic and industrial wastewater (51) and the potential of AGS to adsorb OFL, NOR and OFL has been demonstrated in a laboratory scale bioreactor (51).

1.3.1 Biosorption

Biosorption is the accumulation in a biosurface of the compounds present in a gaseous or liquid phase. The compounds are transferred by physico-chemical processes to the biosurface, that is, there is an accumulation of a substance (biosorbate) at a biological interface (biosorbent) (52). Meanwhile, the process of removing the compound from the surface is called desorption.

Biosorption is considered one of the most promising techniques for wastewater treatment in recent decades (53). This technique has become popular due to its efficiency in removing very stable pollutants, laboratory scale, by biological methods. The economic crisis of the 90s led to an increased interest in biosorbent materials with lower cost and more environmental friendly hence the designation of "green biosorption" (53). This term applies to the use of low cost biosorbent materials from agricultural sources and by-products (fruits, vegetables, food) and other wastes (AS and AGS) that are low cost sources from which most complex biosorbents can be produced (53). These "green biosorbents" may have reduced biosorption capacity in comparison to a super-adsorbent such as activated charcoal which is the most reported in the literature, but also more expensive and difficult to regenerate. Besides the level of cost and the environmental benefits, "green biosorbents" are much more competitive (54). Therefore one of the main advantages of the biosorption process is the possibility of using low cost materials, making the process more economical. Furthermore these biosorbents may be modified before being used for the removal of pollutants thus allowing to increase their biosorption capacity (53).
Selection of the most suitable biosorbent is carried out taking into account some characteristics, such as:

(i) low cost, taking in account the reusability;

(ii) satisfactory biosorption capacity;

(iii) minimal impact on the environmental balance.

After the biosorbents being used, they must be discarded or regenerated for new use, depending on the costs associated with regeneration and the type of pollutant that was adsorbed. In many cases, the used biosorbents are treated as hazardous waste being incinerated which may lead to environmental and social problems (54).

The regeneration of the biosorbent material is an important feature as it limits the use of new virgin biosorbent material, bringing multiple economic, industrial and environmental benefits.

The theoretical principle of biosorption is manly based on the surface binding being the biosorbate physically retained on the biosorbent surface, the increase of the surface will cause greater biosorption efficiency.(27).

The mechanism of binding of the biosorbate to the biosorbent determines the type of biosorption occurred: ion-exchange, adsorption, absorption, complexation and precipitation (52). Depending on the type of phase the biosorbate is present, the biosorbent and the biosorbate, it is possible that some of these mechanisms act simultaneously on several levels (52).

Among the above mentioned mechanisms, the most common explanation for the phenomenon of biosorption is the adsorption (52). The adsorption process is a surface phenomenon involving the attraction and deposition of molecules, normally present in an aqueous phase, in a solid surface (53). The interactions and forces involved in adsorption are mainly electrostatic interactions (*Van der Waals forces*) and hydrogen bonds. Covalent bonds are also possible, but they happen much more rarely. The molecules can adsorb to the solid surface by two ways: forming monolayers or forming multilayers (52). The differences in adsorption monolayer and multilayer adsorption are shown in Figure 5. Since adsorption is ruled by weak interactions, the adsorbed molecules can be desorbed making adsorption an easy reversible phenomenon.

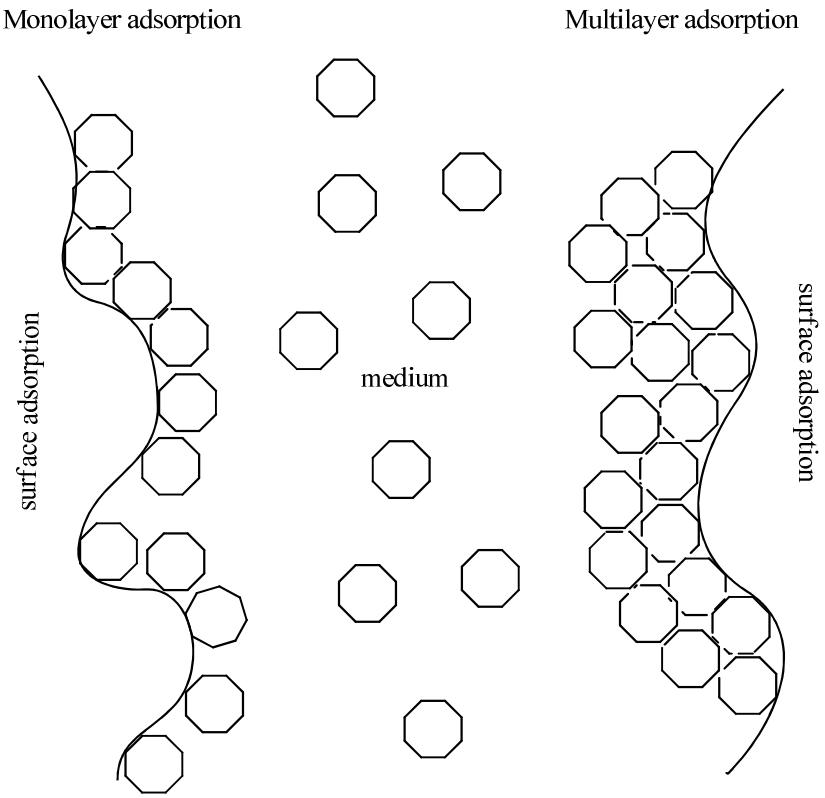


Figure 5. Monolayer and multilayer biosorption .

1.3.1.1 Kinetics and mechanisms of biosorption

The study of the kinetics of a given biosorption process is important, since it provides information on the mechanism of biosorption, in order to evaluate the effectiveness of the procedure.

The biosorption mechanism is complex and can be divided into three steps:

(i) transfer of the solute present in the solution to the surface at solid-liquid interface that encompasses the biosorbent (mass transfer);

(ii) solute diffusion through the pores of the biosorbent (intraparticle diffusion);

(iii) solute binding to the active sites of the biosorbent where the molecules are finally biosorbed by the porous surface of the biosorbent (biosorption).

From the steps mentioned above, biosorption is the faster step of the process, assuming equilibrium is attained when the amount of adsorbed solute on the biosorbent surface is in equilibrium with the concentration of solute in solution. The external transport and / or

intraparticle diffusion are the limiting factors and therefore the main responsible for the overall rate of sorption (55).

The biosorption rate of the solute to biosorbent is described by the biosorption kinetics, whose type allows predicting the rate at which each pollutant is removed from the solution.

The kinetics of biosorption are typically described in the literature for the models of pseudo-first order (56) or pseudo-second order (52). These two models are applied in situations where the dominant phase, that is the slowest step in the biosorption process, is the chemical reaction (mass transfer).

These models admit the existence of a relationship between accumulation of solute in the solid phase (dqt/dt) with a kinetic constant (k) and a driving force that induces progression of the reaction, in other words:

$$\frac{dqt}{dt} = k(q_e - qt)^y \quad (eq.\ 1)$$

where value of the exponent in the equation (γ) provide information about the order of the reaction.

Pseudo-first order model

The model of pseudo-first order (γ = 1) is represented by the following equation:

$$q_t = q_e (1 - e^{-k_1 t}) \quad (eq.\ 2)$$

This equation is a result of the integration of equation 1 given that q and qt corresponding to the biosorption capacity in equilibrium and at the time t, respectively (mg g^{-1}) and k_1 is the rate constant for pseudo first order (L min^{-1})(56).

Pseudo-second order model

The model of the pseudo-second order (γ = 2) is represented by equation 3, which is also originated from the integration of Equation 1 (57):

$$q_t = \frac{q_e^2 k_2 t}{1 + q_e k_2 t} \quad \text{(eq. 3)}$$

where k_2 is the rate constant of the pseudo-second-order (g mg^{-1} min^{-1}). (56).

Apart from these, there are other models described in the literature such as the external diffusion model and the intraparticle model which will not be addressed in this work.

1.3.1.2 Biosorption isotherms

The biosorption isotherms are mathematical expressions that relate the amount of solute in equilibrium, the solid phase (q_e) and the solute concentration in the liquid phase at equilibrium (C_e), with constant temperature.

There are various types of biosorption isotherms, and the most commonly found in the literature are the linear isotherm, the Langmuir isotherm and the Freundlich isotherm (52).

Linear Isotherm

The linear isotherm is the simplest model that describes the accumulation of solute in the biosorbent material as being directly proportional to the equilibrium concentration in the solution.

$$q_e = C_e k_d \quad \text{(eq. 4)}$$

q_e - amount of solute adsorbed (mg g^{-1})

C_e - equilibrium concentration (mg L^{-1})

k_d - distribution coefficient (L g^{-1}).

This model is commonly used for the biosorption of solutes present in low concentrations.

Langmuir Isotherm

The Langmuir isotherm is based on the following assumptions:

(i) the surface of the biosorbent is homogeneous;

(ii) biosorption occurs at specific locations on the biosorbent surface;

(iii) each molecule occupies an active site and no interactions occur between the adsorbed molecules and adjacent places ;

(iv) the energy of biosorption is the same for all biosorption sites;

(v) formation of a unimolecular layer of solute molecules (monolayer)

(vi) biosorption is a reversible phenomenon.

The model can be represented by the following equation:

$$q_e = \frac{q_{max} k_l C_e}{1 + k_l C_e} \qquad \text{(eq. 5)}$$

q_{max} - capacity of the monolayer (mg g^{-1})

k_L - equilibrium constant of biosorption (L mg^{-1}).

Freundlich isotherm

The Freundlich isotherm assumes the existence of local interactions with more affinity which consequently will be occupied first. Thus the interaction strength decreases with increasing the degree of occupancy of such sites with greater affinity (52).
This model is more adjusted to describe biosorption systems on heterogeneous surfaces where the biosorption is reversible and is not restricted to the formation of monolayers (55).

$$q_e = k_f C_e^{1/n} \qquad \text{(eq. 6)}$$

K_f - equilibrium constant of biosorption (mg g^{-1}(mg L^{-1})$^{-1/n}$)

n – Freundlich constant.

The value of n reflects the degree of heterogeneity of the surface, being indicative of the degree of biosorption (n> 1 when the isotherm is favorable and <1 for an unfavorable isotherm).

Besides the models above described, there are other in the literature, which are variations of these models.

1.3.1.3 Factors influencing the biosorption processes

The main factors to be considered in the biosorption process are as follows (52):

(i) characteristics of the biosorbent and the biosorbate;
(ii) the concentration of the pollutant to be removed;
(iii) the quantity of effluent to be treated;
(iv) the amount of biosorbent material;
(v) the pH and temperature;
(vi) the contact time.

One of the most important factors in the biosorption is pH. This can induce modifications on the pollutant under study, or either on the biosorbent surface (58). The effect of pH is particularly important in situations where the compounds and the biosorbent surface present amphoteric biosorption behavior due to ionizable functional groups such as the FQs (58, 59) .
The biosorbent properties such as the surface area, the concentration of extracellular polymeric substances (EPS), the concentration of organic matter, among others, are extremely important in the biosorption process, determining the effectiveness of the method.
The EPS are a mixture of polymers of high molecular weight which appear as metabolic products of microorganisms or by cell lyses and accumulate on the cell surface, forming a protective barrier for the cells against the external environment (60). The composition of EPS is heterogeneous and depends on the type of the microbial aggregates used as biosorbent and may be constituted for proteins, humic substances, nucleic acids, among others (49). These biopolymers have functional groups (e.g. hydroxyl and carboxyl groups) that allow specific interactions such as hydrophobic interactions, hydrogen

bonding, and ionic interactions (61). So the EPS have a significant influence on the physicochemical properties of microbial aggregates, and thus could affect the entire microbial surface and consequently the capacity of aggregation, biosorption, biodegradation, mass transfer and structural stability (60).
Furthermore, the concentration of pollutants and organic matter in the effluent are important factors since the organic matter may compete for the available surface sites on the biosorbent (52).

1.3.2 Adsorption of FQs to biotic and abiotic matrices

Adsorption is a physical process of adsorbate adherence the biotic or abiotic matrices (52). The use of biotic matrices (AS and AGS) allows the pollutants degradation and transform, in addition to biosorption processes. Biotic matrices can be used for specific applications when pollutant removal is not feasible and biodegradation and biotransformation are required (52). However biodegradation has as disadvantage the possibility of producing more toxic metabolites to the environment.

Adsorption has the advantage of removing the substance analyze instead of producing metabolites that may be potentially more dangerous (11). However, this process promotes the production of a new residue by the transfer of pollutant to the adsorbent. This new solid residue (adsorbent + drug), if possible, must be treated for reuse (53).

Table 3 lists some of the adsorption studies with biotic (AS and AGS) and abiotic (zeólite, hentonite and hirnessite) matrices and evaluation of the mechanisms for FQs removal.

Table 3. Adsorption of FQs to biotic and abiotic matrices.

FQs	Adsorbent	Ci (mg L^{-1})	Test conditions	% adsorption (qe (mg g_{TSS}^{-1}))	Reference
Gatifloxacin	Activated sludge	100	0,05 g adsorbent T = 25 ºC	96% (19,8)	(27)
Enrofloxacin	Zeolite	200	pH = 5 T = 28 ºC 0,75 g adsorbent	80% (19,3)	(58)
NOR OFL CPF	Activate sludge	0,1	Reactor average flow of adsorbent = 8,8*103 m^3 T =25 ºC	61% 43% 53%	(56)
CPF	Bentonite	100	pH = 4,5 T = 25 ºC 2,5 g adsorbent	98% (147,1)	(62)
CPF	Birnessite	1000	0,13 g adsorbent	81% (71)	(63)
NOR OFL CPF	Aerobic granular sludge	3	Reactor operating in 8 h cycles, continuous load of FQs at a conc of 3 µM.	(0,012) (0,010) (0,017)	(51)

Although adsorption is a known process, there are few studies on the applicability of this process for the treatment of polluted wastewaters containing FQs.

As can be seen in Table 3 even when the initial concentrations were high, the adsorption method was effective in removing FQs in either biotic or abiotic matrices.

1.4 Objectives

The objective of this work is to study the biosorption of three FQs: OFL, NOR and CPF, in two different biosorbents, AS and AGS.

The FQs are fluorinated antibiotics, suggesting a great potential for environmental persistence. OFL, NOR and CPF are widely used in human and veterinary medicine. Furthermore, FQs have been detected in various environmental matrices such as hospital and industrial effluents, WWTP, surface water, soils, food and even in drinking water. The choice of biosorbents took into consideration the needed for a green procedure and the importance of the costs associated with the removal process and the environment protection. Studies to evaluate mechanisms for removing micropollutants with AS and AGS demonstrated that such biosorbents are effective in the removal process.

Therefore, to achieve the objectives referred above the following described work was performed:

- To validate the analytical method for quantification of OFL, NOR and CPF by High performance liquid chromatography with fluorescence detection (HPLC - FD);

- To characterize the studied biosorbents namely, AS and AGS;

- To evaluate the effect of the amount of biosorbent on the biosorption profile in AS;

- To assess the effects of the initial concentration of FQs and the contact time on the biosorption profile in AS and AGS;

- To determine the biosorption kinetics and isotherms for OFL, NOR and CPF in AS and AGS;

- To determine the degree of desorption of OFL, NOR and CPF in AGS by changing the pH of the medium.

2. Material and Methods

2. Material and Methods

2.1 Determination of the degree of ionization of the OFL, NOR and CPF at different pH

Calculations of the degree of ionization and the presence of charged microspecies, of the OFL, NOR and CPF, depending on the pH were performed using MarvinSketch 14.8.18.0 software from ChemAxon.

2.2 Analytical methods for measurement of OFL, NOR and CPF

HPLC - FD was used for the quantification of the target FQs (OFL, NOR and CPF). The analytical method was based on previously work developed by the research group (64).

2.2.1 Solvents, antibiotics and stock solutions

Ethanol (HPLC grade) was purchased from Carlo Erba Reagents. Triethylamine with ≥ 99% purity was obtained from Sigma-Aldrich. Acetic acid and trifluoroacetic acid were purchased from Panreac (≥ 99% purity) and Acros Organics (≥ 99% purity), respectively. The ultra-pure water was obtained from a Milli-Q system.
The OFL, NOR and CPF antibiotics were purchased from Sigma (98% purity). Individual stock solutions of OFL, NOR and CPF 10 mg mL^{-1} were prepared in water/acetic acid 10% (50:50 *V/V*). These solutions were stored at -20 °C in amber bottles. Stock solutions of the three FQs at 10 mg mL^{-1} were also prepared. From this stock solution, standard solutions of the three FQs in different concentrations were prepared. All standard solutions were prepared weekly by dilution of the stock solutions in mineral medium. All reagents used in this study were analytical – grade chemicals.

2.2.2 Sample preparation

AS and AGS were used as biosorbents in this study mixed in minimal medium. The chemical composition of the medium solution was: $NaCH_3COO$, 0.48 g; $MgSO_4.7H_2O$,

0.08 g; KCl, 0.03 g; Na_2HPO_4, 0.06 g; KH_2PO_4, 0.03 g; NH_4Cl, 0.18 g and 0.9 mL of trace elements (composition per liter: 63.77 g EDTA.$2H_2O$; 22 g $ZnSO_4.7H_2O$; $CaCl_2$ 5.54 g; $MnCl_2.4H_2O$ 5.06 g; 4.99 g $FeSO_4.7H_2O$; (NH_4) $6Mo_7O_{24}.4H_2O$ 1.1 g; $CuSO_4.5H_2O$ 1.57 g; $CoCl_2$ 0.88 g; KOH to adjust to pH 6). Samples were collected and centrifuged for 10 minutes at 14000 rpm and then the supernatant was acidified with glacial acetic acid (1:100) to pH 3. All samples were analyzed by the validated HPLC-FD method.

2.2.3 Equipment

The HPLC was a Finnigan Surveyor (Thermo Electron Corporation, USA) equipped with an autosampler (AutoSampler Plus) and a fluorescence detector UltiMate FLD3100 (Thermo Scientific Dionex, USA). The column was a Luna PFP (2), pore size 100 Å, particle size 3 µm, 150 x 4.6 mm, a modified reverse phase silica-based column, from Phenomenex. The mobile phase consisted in mixture of ultra-pure water with 0.1% TEA and TFA (pH 2.2) and ethanol 74:26 (*v/v*). The flow rate was set to 0.7 mL min^{-1}. The injection volume was 10 µL and the column oven temperature was 45 ºC. The fluorescence detector was set to an excitation wavelength of 290 nm and an emission wavelength of 460 nm. The software used for the treatment of the chromatographic data was the Chromeleon software 7.1 SR2 (Thermo Fisher Scientific).

2.2.4 HPLC-FD method validation

The HPLC-FD method was validated according to Q2B ICH (1996) and FDA (2001). All parameters were evaluated for each FQ (65, 66).

a) Selectivity

The method selectivity was studied using the matrixes of biosorption assays: AGS and AS, mixed, separately, in mineral medium. Aliquots of 1 mL were collected at 15 and 120 minutes and centrifuged for 10 minutes at 4 °C and 14 000 rpm. The supernatant was acidified with acetic acid (1:100) to pH 3.0 and subsequently analyzed by HPLC-FD. The resulting chromatogram was compared with the chromatogram resulting from the injection of a standard solution of the three FQs (400 ng mL^{-1}) and with the chromatogram resulting

from the injections of AGS matrix or AS matrix enriched with each FQ at final concentrations of 300 ng mL^{-1} and 200 ng mL^{-1}.

b) Linearity and range

Calibration curves were performed with nine different concentrations of the standard solution of the three FQs prepared by dilution of the stock solutions in mineral medium (pH 7): 0, 10, 25, 50, 100, 250, 400, 600 and 800 ng mL^{-1} of each FQ. Five replicates for each concentration were prepared and each replica was analyzed in triplicate.

The calibration curves were obtained by linear regression corresponding to the correlation between the peak area and the nominal concentration.

c) Detection and quantification limits

The detection limits (DL) and quantification limits (QL) were determined based on the signal / noise ratio (S / N). Determination of S/N was performed by comparing measured signals from samples with low substance analyze concentrations with those of blank samples and establishing the minimum concentration at which the substance analyze can be reliably detected or quantified. S/N ratios of 3:1 and 10:1 are acceptable to estimate the DL and QL, respectively (67).

d) Precision

Precision was evaluated taking into account two factors, the repeatability and the intermediate precision. To evaluate repeatability, the relative standard deviation (RSD) of nine determinations of the FQs standard solution was analyzed in the same conditions and on the same day. To analyze the intermediate precision, the relative standard deviation of six determinations of the same FQs standard solution in three consecutive days was determined.

In this study, three different concentrations of the standard solution of the three FQs (4, 450 and 650 ng mL^{-1} of each FQ) were used. Three replicates for each concentration were prepared and each replica was analyzed in triplicate.

e) Accuracy

Accuracy was evaluated using three different concentrations of the standard solution of the three FQs (4, 450 and 650 g mL^{-1} of each FQ) were used. Three replicates for each concentration were prepared and each replica was analyzed in triplicate. The determination was made based on the ratio between the concentration given by the peak area of each FQ in the standard solutions and the nominal concentrations.

f) Stability

The chemical stability of OFL, NOR and CPF was assessed in standard solutions and in samples, taking into account the analysis conditions and keeping (20 °C and -20 °C).Standard solutions were prepared containing the three FQs at the concentrations of 4, 450 and 600 ng mL^{-1} of each FQ. The samples corresponded to the supernatant of the result from the 3 h contact, of OFL, NOR and CPF, with AS in mineral medium. These samples concentrations were 8, 380 and 780 ng mL-1 of each FQ. Stability at -20 °C was evaluated after three cycles of freeze/defreeze at 24h, 48h and 72h. Stability at 20 °C was evaluated at 0h, 24h and 48h, wherein each analysis interval between the standard and sample solutions were maintained within the autosampler, to ensure that always maintain the same temperature.

2.3 Biosorption studies

2.3.1 Preparation of biosorbents

The biosorption studies were conducted using AS and AGS (≥ 0.45 μm) as biosorbents (Fig. 6). The AS was collected from the aeration tanks of a municipal WWTP (Ponte de Moreira, Maia - Portugal). The AGS was collected from a laboratory scale sequential batch reactor. In order to remove impurities, AS was washed two times with saline solution (0.85% w/v NaCl) and suspended in mineral medium for the biosorption experiments.

AGS, after being collected from the reactor was washed twice with saline solution and was sieved through a 0.45 μm pore size sieve. The AGS weas then homogenized in mineral medium and used in the biosorption experiments.

To evaluate the biosorption capacity of the inactivated biosorbents, after the washing and homogenization process in mineral medium, AGS and AS were autoclaved at 200 °C for 90 minutes.

All the experiments were performed in duplicate under sterile conditions and protected from light.

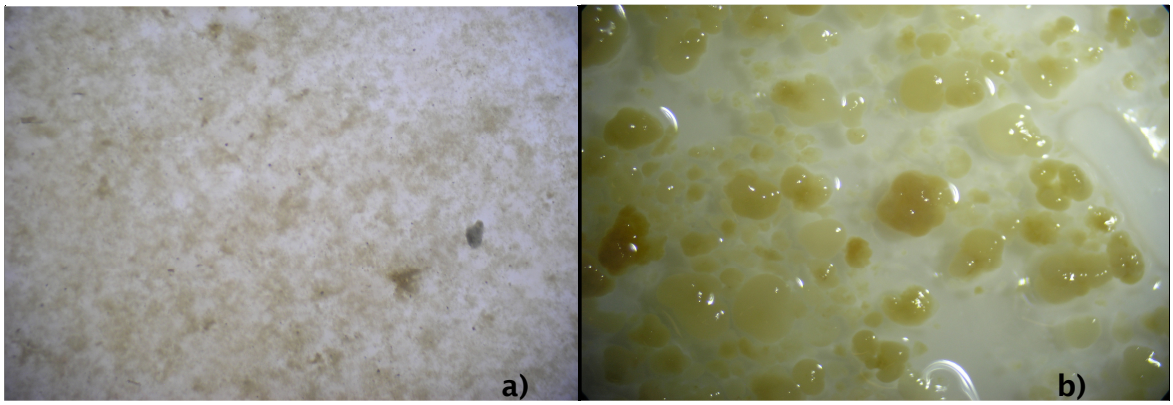


Figure 6. Microscopic aspect of biosorbent: a) AS; b) AGS.

2.3.2 Determination of EPS

The EPS content on both biosorbents (AGS and AS) was analyzed. The EPS was extracted using a heat method according to the previous research (68, 69). A schematic representation of the methodology is shown in Fig. 7. The extraction method used was similar for both biosorbents under study.

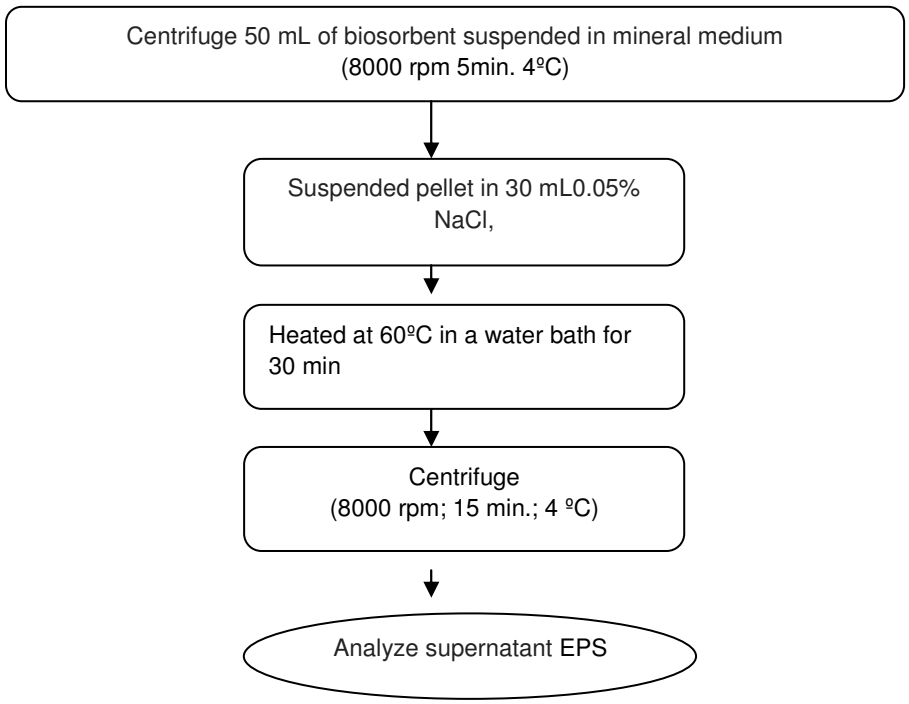


Figure 7. Schematic representation of the methodology for extracting EPS.

The carbohydrate content in EPS was estimated by anthrone method (67) using glucose (Merck) as standard. Briefly, 0.8 mL of sample/standard was mixed with 1.6 mL of 0,125% anthrone (w/v) in 94.5% (v/v) H_2SO_4 reagent. Samples were placed in a water bath at 100°C for 14 min and then cooled at 4 °C for 5 min. Absorbance at 625 nm was measured.

The protein content in EPS was measured using the Lowry method (Fig 8) (67). BSA was used as standard.

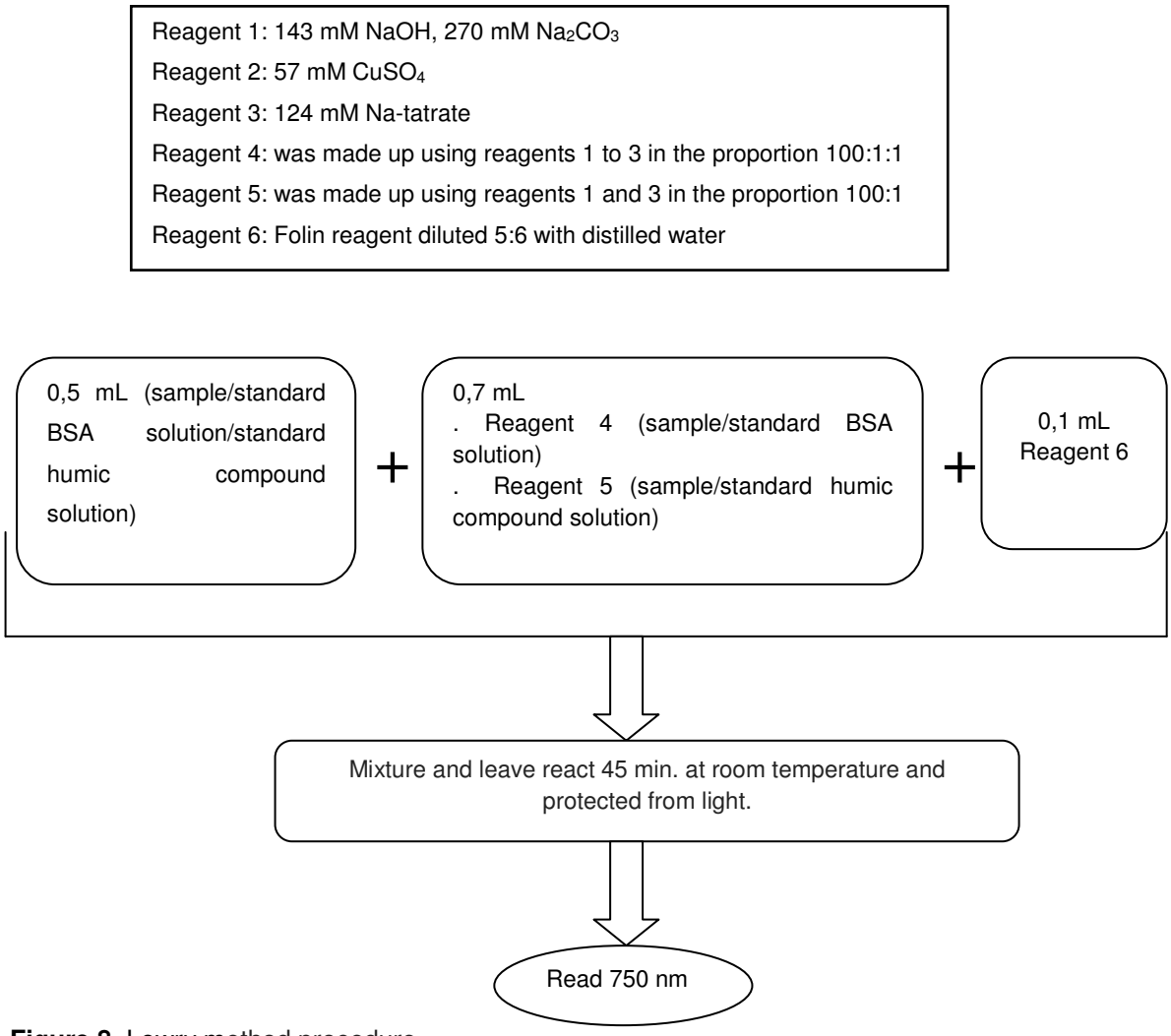


Figure 8. Lowry method procedure.

A modified Lowry method was applied to determine the humic substance content by correcting the protein interference. Humic substances interfere with the Lowry procedure (67). A correction for interference of humic compounds, as described by Frolund *et al.* (1995), was made by the following equations (70):

$$A_{total} = A_{protein} + A_{humic} \quad (eq.7)$$

$$A_{blind} = 0.2\ A_{protein} + A_{humic} \quad (eq.8)$$

$$A_{protein} = 1.25\ (A_{total} - A_{blind}) \quad (eq.9)$$

$$A_{humic} = A_{blind} - 0.2\ A_{protein} \quad (eq.10)$$

where A_{total} is the total absorbance with $CuSO_4$, A_{blind} is the total absorbance without CuSO4, A_{humic} is the absorbance due to humic compounds, and $A_{protein}$ is the absorbance due to proteins. All assays for biosorbent characterization were performed in duplicate.

2.3.3 Determination of Zeta Potential

To determine the zeta potential, the AS and AGS were homogenized in mineral medium at pH 7 and analyzed by a ZetaPals (Brookhaven, Zeta Potential Analizer). The initial pH, of the same sample of AGS, was adjusted to the desired pH value in the range of 2-9 with hydrochloric acid (0.5 M HCl) and sodium hydroxide (0.1 M NaOH) solutions. The sample was analyzed after 15 min. of pH adjustment.

2.3.4 Biosorption experiments

In this work the biosorption capacity of AS and AGS towards FQ antibiotics (OFL, NOR and CPF) in aqueous matrix was evaluated. The experiments were conducted in 250 mL glass flasks containing 75 mL of mineral medium supplemented with a mixture of three FQs. The flasks were incubated at 25 ºC on a rotary shaker (Stuart Incubator Model Si500) (130 rpm). The schematic representation of the biosorption experiment is presented in Figure 9. All the experiments were performed in duplicate under sterile conditions. Samples were taken at regular intervals and were centrifuged at 14 000 rpm (Hettich Zentrifugen model Universal 320R) for 10 min at 4 °C. The supernatant was collected, acidified with glacial acetic acid (1:100) to pH 3 and stored at -20 °C protected from light until analyzed by HPLC-FD.

The substance analyze concentration retained in the biosorbent was evaluated using the following equation:

$$q = \frac{(C_0 - C)V}{m} \qquad \text{(eq. 11)}$$

where *q* is the amount of each compound adsorbed (mg g_{TSS}^{-1}); C_0 and C is the concentration of each compound at the beginning and at time t (ng mL^{-1}), respectively; V is the volume of the aqueous phase (mL) and m is the mass of biosorbent used (g). The percentage of removal of each compound was determined using the following equation:

$$\% \text{ biosorption} = \frac{(C_0 - C)\ 100}{C_0} \qquad \text{(eq. 12)}$$

The total suspended solids (TSS) and volatile suspended solids (VSS) were determined according to Standard Methods for the examination of Water and Wastewater (71).

2.3.5 Kinetics of biosorption

For the kinetic assays 10 mg_{TSS} of each biosorbent were inoculated in 75 mL of mineral medium containing different concentrations of each FQ within the range of 100 and 700 ng mL^{-1}.

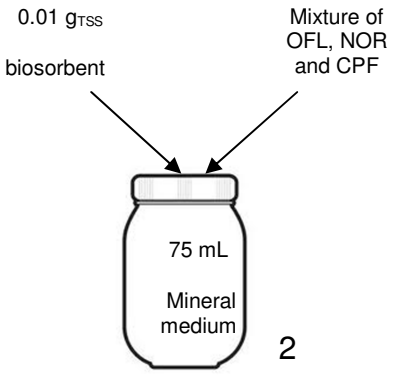


Figure 9. Schematic representation of the biosorption assays.

Samples were collected at time 0; 0.75; 1.5; 2.25; 3; 24 and 48 h for analysis. During the experiment the flasks were in permanent agitation (120 rpm) in an orbital shaker, at constant temperature (25 °C) and protected from light. After 48 h, the suspension was filtered through a membrane of glass fiber GF/C with a porosity of 1.2 µm to determine TSS.

In parallel, abiotic controls (without addition of biosorbent) exposed to the same experimental conditions were carried out. All assays were performed in duplicate and samples were kept at -20 °C until analyzed by HPLC-FD.

2.3.6 Biosorption isotherms

The procedure was analogous to that described for the determination of the kinetics of biosorption, and this time the samples were taken only at 24h. Assays were performed in duplicate and samples were kept at -20 ° C and protected from light until analyzed by HPLC-FD.

2.3.7 Desorption assays with AGS

In order to study the desorption process of the AGS after being exposed for 48h to a mixture of FQs, the initial pH of each solution was adjusted to the desired pH value in the range of 3 - 9 with hydrochloric acid (HCl 0.5 M) or sodium hydroxide (NaOH 0.1 M) solutions. Samples were collected at 15, 30, 45 and 60 minutes after the pH being adjusted.

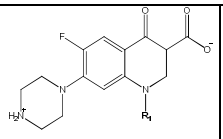
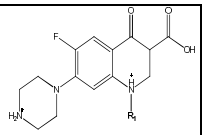
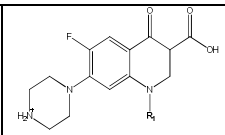
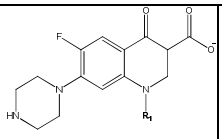
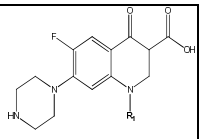
3. Results and Discussion

3. Results and Discussion

3.1 Determination of the degree of ionization of the OFL, NOR and CPF at different pH

Calculations of the degree of ionization and the presence of charged microspecies, of the OFL, NOR and CPF, depending on the pH were performed using MarvinSketch 14.8.18.0 software from ChemAxon. The results are shown in Table 4.

Table 4. Degree of ionization and the presence of charged microspecies of the OFL, NOR and CPF at different pH

FQS	pH					
OFL	2	0.03	Does not present this form ionic	99.96	0	0.01
	3	0.27		99.64	0	0.08
	4	2.63		96.53	0.02	0.82
	5	19.77		1.63	72.48	6.12
	6	40.01		14.67	32.94	12.38
	7	10.44		0.38	85.95	3.23
	8	1.2		0	98.43	0.37
	9	0.12		0	99.84	0.04
NOR	2	0.02	0.88	99.1	0	0
	3	0.17	0.09	99.74	0	0
	4	1.67	0.01	98.32	0	0
	5	14.53	0	85.45	0	0.02
	6	62.84	0	36.95	0.13	0.08
	7	92.5	0	5.44	1.94	0.12
	8	82.16	0	0.48	17.26	0.1
	9	32.23	0	0.02	67.71	0.04
CPF	2	0.02	0.6	99.39	0	0
	3	0.17	0.06	99.76	0	0
	4	1.72	0.01	98.27	0	0
	5	14.92	0	85.06	0	0.2
	6	63.55	0	36.24	0.13	0.8
	7	92.66	0	5.28	1.95	0.11
	8	82.17	0	0.47	17.27	0.1
	9	32.23	0	0.02	67.72	0.04

3.2 HPLC-FD method for quantification of OFL, NOR and CPF

3.2.1 Method optimization

The HPLC - FD method aimed the chromatographic separation of OFL, NOR and CPF present in various samples, in the shortest time and with minimum of interferences. The FD detection allowed the quantification in low level of concentration and without matrix interferences.

The HPLC - FD method was based on work published elsewhere (64). These adjustments have undergone minor changes in the proportions of each eluent: mixture of ultra-pure water with 0.1% triethylamine and trifluoroacetic acid (pH 2.2) and ethanol 74:26 (*v/v*), on the column oven temperature (45 °C) and flow of mobile phase (0.7 mL min^{-1}). The increase of the column oven temperature and the flow of mobile phase was aimed at decreasing the time of chromatographic analysis. These changes also improved the chromatographic separation of FQs, as well as resolution and peak symmetry. OFL, NOR and CPF were eluted with proper separation in 12 minutes. The peaks showed adequate resolution and good symmetry (Figure 11).

3.2.2 Validation of analytical method

The HPLC-FD method was validated according to Q2B ICH (1996) and FDA (2001).
To validate the analytical method five parameters were studied: selectivity, to prove the absence of interfering; linearity, which verified the proportionality between the concentration of each substance analyze and the area of the respective chromatographic peak; accuracy, which allows to verify the agreement between the result obtained with a sample and the true value; precision, to evaluate the dispersion of results between independent and repeated tests of the same sample and the stability of samples at different temperatures over time.

a) Selectivity

Selectivity is the ability to assess unequivocally the target substance, in the presence of other components (impurities, degradation products, matrix interferences, among others).

To study the selectivity of the developed method aliquots of the matrix supernatant of mineral medium and AGS or AS from the biosorption tests were analyzed.

The chromatograms of the matrices in the absence of the target substance (Fig. 10) showed the absence of interferences, thus confirming the good selectivity of the analytical method.

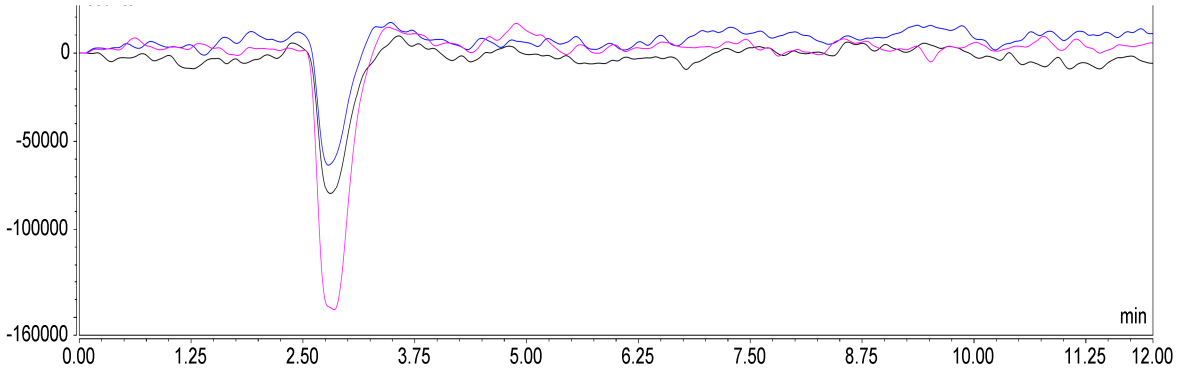


Figure 10. Chromatograms of the matrices: mineral medium (blue line); mineral medium with AS (black line); mineral medium with AGS (pink line).

The chromatograms resulting from the analysis of the matrices were compared with chromatograms resulting from the analysis of standards and samples. Figure 11 shows the chromatogram of a standard solution concentration with 400 ng mL^{-1} of each FQ (a), a sample containing AS with 200 ng mL^{-1} of each FQ (b), and a sample of AGS with 300 ng mL^{-1} of each FQ (c).The comparison between different chromatograms presented allowed to conclude the absence of matrix interferences .

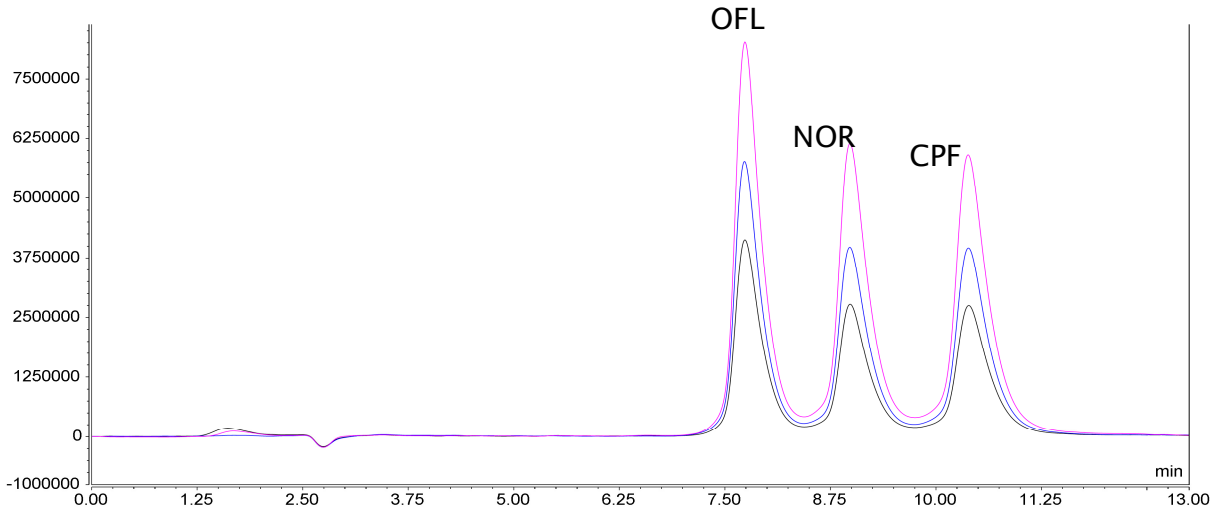


Figure 11. Chromatogram of: a standard solution with a mixtureof each FQ (400 ng mL^{-1}) (pink line); a sample of AGS with 300 ng mL^{-1} each FQ in the mixture (blue line); a sample of AS with 200 ng mL^{-1} each FQ in the mixture (black line).

b) Linearity and range

The linearity is the ability of the analytical method to generate results that are proportional to the concentration of target substance, within a given range, being possible to relate the result with the concentration detected. According to ICH (1996) a minimum of five different concentration levels are needed to demonstrate the linearity within the desired range (65) and the results should be treated with appropriate statistical methods such as linear regression. The variation of the linear response of this method was conducted in an appropriate range of concentrations and the linearity was confirmed. Calibration curves performed over the established concentration range (0 - 800 ng mL^{-1}) are presented in Figure 12.

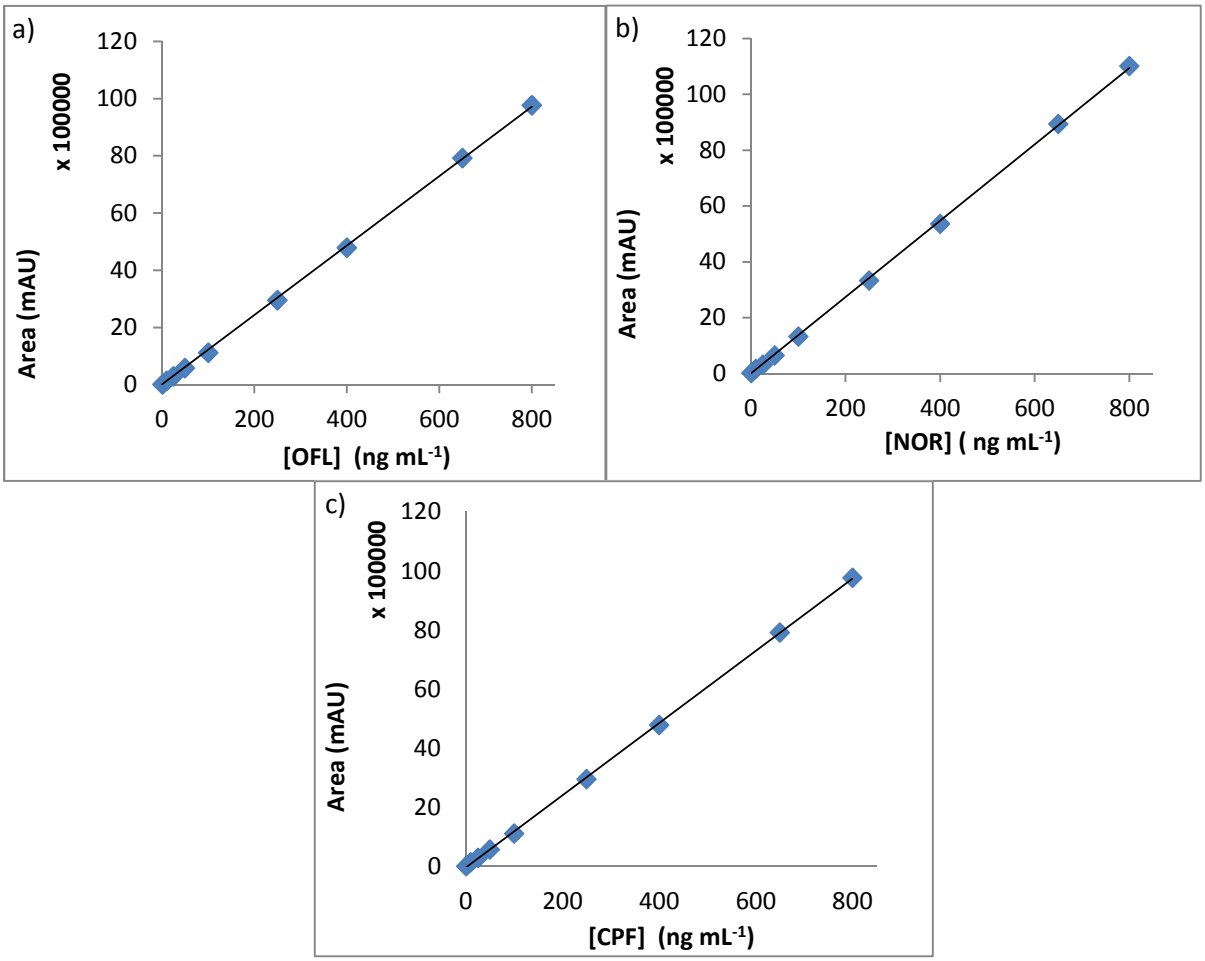


Figure 12. Calibration curves of OFL (a), NOR (b) and CPF (c) in mineral medium.

According to international guidelines for validating analytical methods, the correlation coefficient should be greater than 0.999 to admit that a method is suitable to be used in the analysis (65). In this study, correlation coefficients always greater then 0,9997 were

obtained and as such it can be stated that these calibration curves are suitable for use in analysis.

c) Detection and quantification limits

The DL of the analytical method is the lowest concentration of substance analyze in a sample that can be detected but not necessarily quantified as an exact value.

The QL is the lowest concentration of substance analyze in a sample that can be quantitatively determined with suitable precision and accuracy.

In this work, the DL and QL were calculated by the signal to noise ratio method (S/N). According Q2B ICH (1996) the determination the S/N ratio is performed by comparing measured signals from known samples with low concentrations of substance analyze with those of blank samples and establishing the minimum concentration at which the substance analyze can be detected safely. The S/N ratio of 3:1 is considered acceptable for estimating the DL. The S/N ratio of 10:1 is adequate to estimate the QL (65) . The values of DL (between 0.6 and 0.7 ng mL^{-1}) and QL shown in Table 5 are suitable to monitoring the target fluorinated antibiotics in the biosorption assays.

Table 5. Linearity, DL and QL

FQs	Calibration curves				Calculation S/N	
	Linearity (ng mL^{-1})	**Slope**	**Zero-intercept**	**r^2**	**DL (ng mL^{-1})**	**QL (ng mL^{-1})**
CPF	2 a 800	13677	0	0.9997	0.6	1
NOR		13672		0,9998		
OFL		12141		1	0.7	

Figure 13 shows three chromatograms corresponding to the DL and QL.

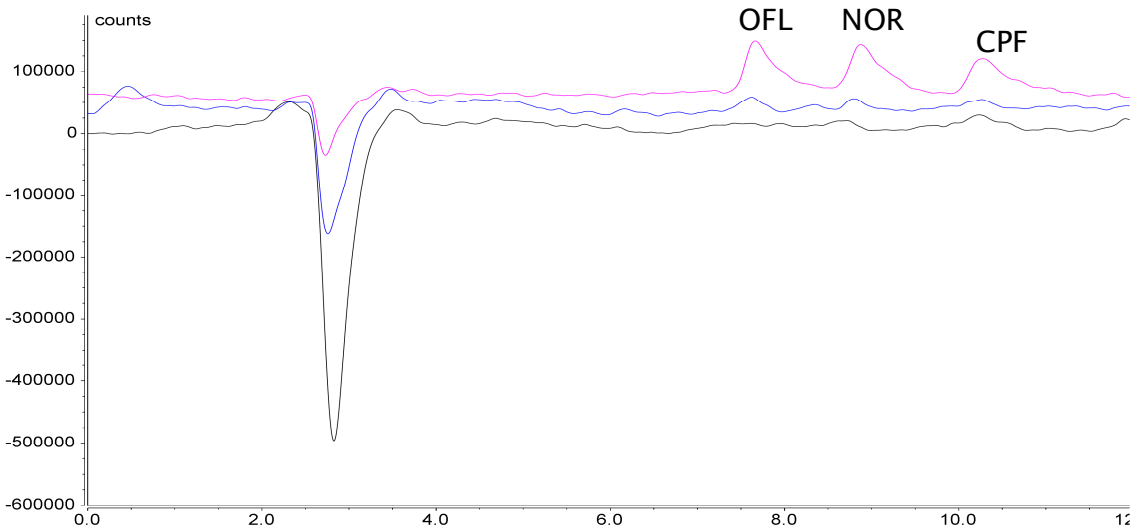


Figure 13. Chromatogram of the standard solution with concentration each FQ: 1 ng mL^{-1} (pink line); 0.7 ng mL^{-} (blue line); 0.6 ng mL^{-1}(black line).

d) Precision

The precision is defined as the degree of similarity between the results for the same sample. Intermediate precision and repeatability were determined through the relative standard deviation and were represented by the RSD (Table 6).

According to the guidelines for validating analytical methods %RSD values up to 15% are considered acceptable taking into account the method employed and the range of the sample concentrations (in the order of ng mL^{-1}) (66). Thus the results indicate that the method is precision.

e) Accuracy

The accuracy of an analytical method expresses the correlation between a given value by the analytical method and the true amount of substance analyze in the sample. According to the FDA (2001), accuracy should be evaluated with a minimum of three different concentration levels in triplicate (total of nine determinations) (66).

In this work we used standard solutions with concentrations, of each FQs in the mixture, of 4, 450 and 650 ng mL^{-1}. The obtained results are shown in Table 6. According to the FDA (2001) values for accuracy should be within in the range of 70% to 130% (65).

Table 6. Precision and accuracy of the analytical method with standard solutions.

FQs	Concentration (ng mL^{-1})	1st day		2nd day		3rd day	
		Accuracy (%)	RSD(%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD(%)
OFL	4	103.1	3.7	105.8	3.9	103.0	4.5
	450	97.5	0.7	96.4	0.7	96.6	0.5
	650	108.9	0.9	104.4	1.7	104.7	1.1
NOR	4	109.9	2.8	106.7	3.9	105.7	2.9
	450	96.5	0.7	95.3	1.1	94.7	0.5
	600	101.4	1.7	101.4	1.1	102.8	0.8
CPF	4	104.2	3.2	108.5	2.6	108.0	3.8
	450	96.5	0.5	95.7	0.5	95.2	0.4
	600	100.4	1.1	101.9	1.2	103.6	1.4

Percentages of accuracy were between 95 and 109%, for the three substance under study. According to the guidelines and it was possible to confirm the accuracy of the method.

f) Stability

The chemical stability of CPF, OFL and NOR was assessed in different environments of study (standard solutions and samples) , taking into account the conditions for the assays of biosorption (20 ° C and -20 ° C) and the respective results are shown in Table 7.

Table 7. Stability tests at – 20ºC and 20ºC

Solutions	Days	Concentration (ng mL^{-1})	Stability 20ºC						Stability - 20ºC					
			OFL		NOR		CPF		OFL		NOR		CPF	
			Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)
Standard solutions	1st	4	101.0	2.2	101.3	2.9	100.3	2.6	99.3	1.3	102.6	1.0	103.3	1.3
	2nd	450	96.2	1.5	104.9	0.5	101.0	0.9	100.61	1.5	100.4	1.3	102.1	1.6
	3rd	650	95.4	0.8	103.1	1.0	100.1	1.7	100.5	0.9	101.0	1.1	103.7	1.5
	1st	4	89.5	2.0	101.3	2.2	100.7	2.9	100.7	0.8	102.4	2.0	102.3	1.6
	2nd	450	92.6	0.7	102.9	0.8	99.4	0.9	99.8	0.5	100.1	1.4	100.4	0.9
	3rd	650	98.4	1.6	101.96	1.5	99.6	1.1	99.6	1.3	100.8	1.2	101.7	1.3
	1st	4	100.5	1.9	101.1	2.5	100.2	2.9	99.5	1.9	103.1	1.7	102.7	1.5
	2nd	450	99.5	0.6	103.9	0.7	101.9	1.1	100.9	1.6	100.8	1.6	101.9	1.1
	3rd	650	97.3	1.5	102.9	1.6	102.1	1.4	98.9	0.9	101.5	0.9	101.5	1.6
Samples	1st	4	99.4	0.6	101.1	1.7	98.5	1.6	100.5	1.4	102.2	1.7	101.8	1.7
	2nd	450	97.3	0.7	89.8	0.8	99.3	1.9	102.6	1.8	101.7	2.4	100.2	2.2
	3rd	650	95.5	0.9	87.6	2.1	101.0	1.4	99.2	0.4	95.9	0.9	96.7	0.5
	1st	4	100.3	0.8	100.4	1.8	98.8	1.3	101.6	1.4	101.9	1.2	100.9	1.4
	2nd	450	101.5	0.9	86.3	1.7	99.8	1.4	100.7	1.6	100.7	1.9	101.1	2.2
	3rd	650	99.1	0.6	89.5	0.4	100.1	1.3	96.7	0.9	97.3	1.8	96.9	0.7
	1st	4	98.7	1.0	99.7	2.7	99.9	2.0	102.8	0.5	101.2	1.3	101.0	1.7
	2nd	450	100.1	1.5	85.8	0.7	101.9	1.9	101.3	2.1	101.7	1.5	103.2	2.1
	3rd	650	89.9	0.9	89.4	1.7	101.1	1.6	98.5	0.5	96.4	98.5	97.1	0.7

Regarding the values determined by the guidelines as acceptable for precision (<15) and accuracy (range of 70% to 130%) (66) it was possible to confirm the stability of the standard solutions and samples at the keeping temperatures (– 20ºC and 20ºC) in three consecutive days.

3.3 Biosorption assays

3.3.1 Characterization of biosorbent

For the characterization of the used biosorbents the concentration of EPS and zeta potential were determined.

3.3.1.1 Determination of EPS

EPS are a mixture of polymers of high molecular weight which accumulate on the biosorbentl surface and are important for formation and maintenance of the tri-dimensional matrix through crosslinkage with multivalent cations and hydrophobic interactions, which ideally serve as a natural ligand source, and provide binding sites for other charged particles/molecules (61).

The concentration of ESP was determined in the two biosorbents, namely AS and AGS. The levels of EPS were slightly higher in AS (83 mg g_{TSS}^{-1}) than AGS (68 mg g_{TSS}^{-1}). EPS can affect the surface charge and hydrophobicity of microbial cells in biosorbent (59). Higher amount of EPS indicate higher amount of high molecular weight polymers composed by proteins, humic compounds, and other polymers with ionizable functional groups (e.g. carboxyl and hydroxyl) (72). These ionizable functional groups correspond to possible biosorption sites and as such higher number of these groups will possibly contribute to higher biosorption capacity.

The constitution of EPS, of each biosorbent, was also analyzed and the obtained results are shown in Figure 14.

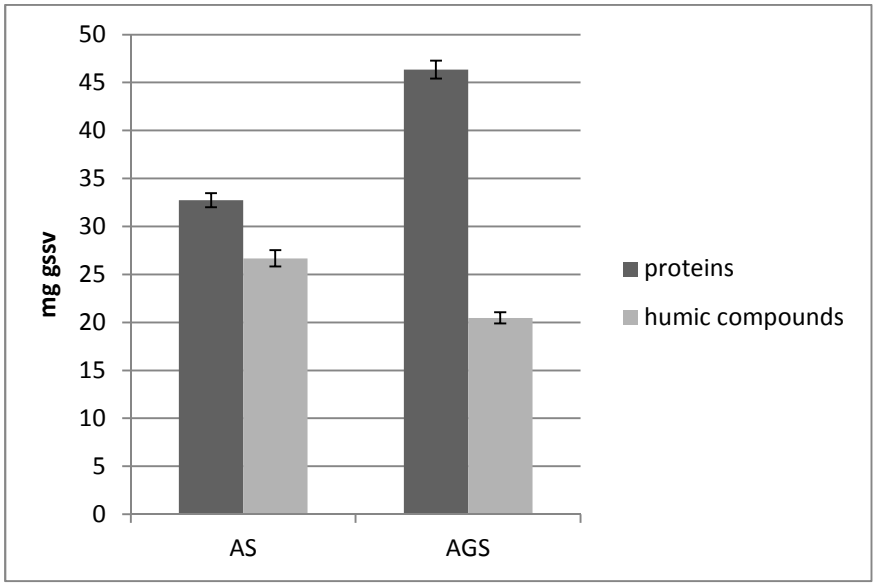


Figure 14. Constitution of EPS of each biosorbent studied.

In both biosorbents it was observed that the protein concentration is higher than the concentration of humic compounds. However is in AGS that there is a greater concentration of protein (47 mg g_{TSS}^{-1}) compared with AS (33 mg g_{TSS}^{-1}).
Many studies reported that the proteins in EPS contribute greatly to the strength and stability of granular sludge, and the decrease of proteins biosynthesis could lead to the aggregates disintegration (61). Furthermore proteins have an high content of negatively charged at amino acids, is being more involved than sugars in electrostatic bonds with multivalent cations, thus decreasing the negative charge at the biosorbent surface.(72).

3.3.1.2 Determination of Zeta Potential

To characterize the biosorbents other parameters such the zeta potential of AGS at different pH was determined. Zeta potential is one of the most useful parameters to characterize the surface charge of the biosorbent. The initial pH, of the same sample of AGS, was adjusted to the desired pH value in the range of 2-9 with hydrochloric acid (0.5 M HCl) or sodium hydroxide (0.1 M NaOH) solutions. All the samples were analyzed after 15 min. of being in contact with the selected pH. Figure 15 showed an inverse relationship between the zeta potential of AGS and the pH of the medium.

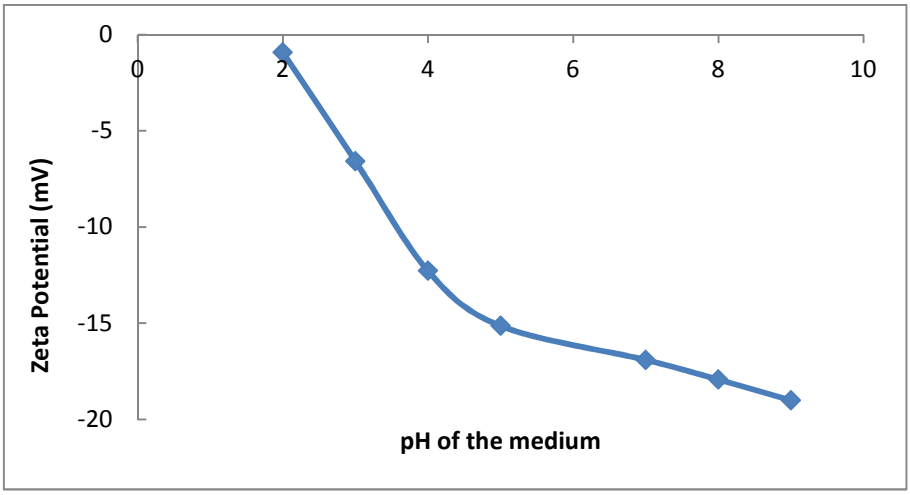


Figure 15 . Zeta potential of AGS at different pH values.

It can be seen that as the pH increases, the zeta potential decreases, thus an increase of the negative charge at the surface AGS was observed.

The zeta potential of AS at pH 7 was also determined which had a value of - 25.65 mV, being higher than the that of AGS in the same condition (-16.21 mV). Zhang (2007) and co-workers also showed higher zeta potential (measured at pH 7) for AS (- 32.4 mV) compared to AGS (-13.3 mV). This study established a relationship between the concentration of the proteins in the biosorbent surface and the zeta potential. As such, the higher zeta potential value in AGS, may be due to the higher concentration of proteins present on the surface biosorbent.

3.3.2 Effect of biosorbent mass in biosorption process

Initially the biosorption of OFL, NOR and CPF was studied in aqueous matrix using AS as biosorbent. Different masses of AS (ranging from 0.01 to 0.3g_{TSS}) were used, keeping all other factors constant. The same test was subsequently performed under the same conditions using the inactive biosorbent. In Figure 16 the results of this assay are shown.

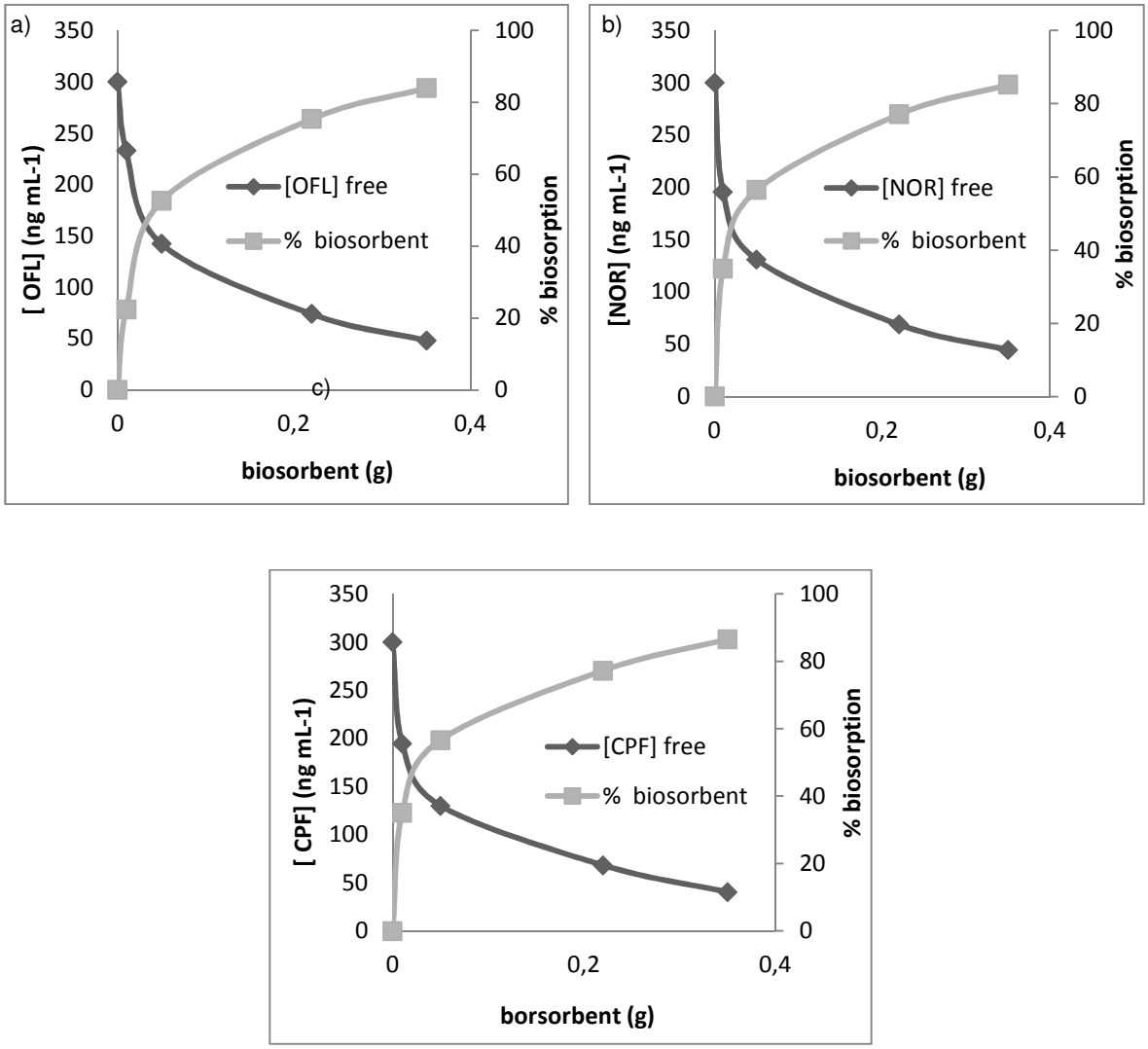


Figure 16. Relation between the initial mass of AS with free concentration and % of biosorption for: a) OFL, b) NOR and c) CPF (time = 24 h, initial concentration of each FQ = 300 ng mL^{-1}, 120 rpm).

The results showed that, for the same initial concentration of analyze substance, when increasing the biosorbent mass there was an increase in the biosorption % of OFL, NOR and CPF, corresponding to the decrease in free concentration of each FQ, in the medium (Fig. 16). This was an expected result since with the increase of the biosorbent mass, the surface biosorbent also increased, promoting the biosorption of the antibiotics.

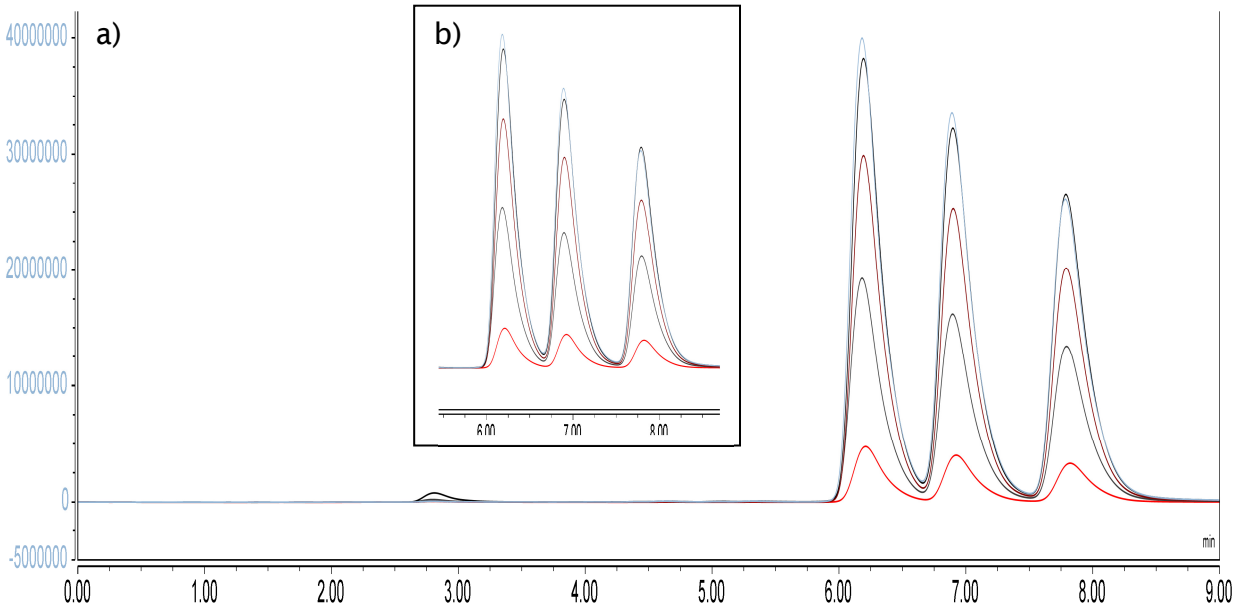


Figure 17. Chromatograms of free concentrations of OFL, NOR and CPF, at 24h, in different mass of biosorbent a) activated or b) inactivated (0 g biosorbent (blue line); 0.01 g biosorbent (black line); 0.05 g biosorbent (brown line); 0.22 g biosorbent (gray line); 0.35 g biosorbent (red line)).

It is possible to verify in chromatograms (Fig. 17) that the results were similar when using activated biosorbent or inactivated biosorbent, which allowed us to conclude that biodegradation does not occur, being the dominant phenomenon the biosorption.

3.3.3 Effect of contact time and initial concentration of OFL, NOR and CPF

This study also evaluated the time of contact needed to reach equilibrium that is, the time required for the biosorbed substance concentration in the system does not vary. The biosorbed amount by AS and AGS was determined as a function of contact time and initial concentration of FQ in each study. The results are shown in Table 8.

Table 8. Effect of contact time on the biosorption process with different Ci (100, 300, 700 ng mL^{-1}) from OFL, NOR and CPF in AS and AGS.

	q (mg g_{TSS}^{-1})											
	Time (h)	100 ng mL^{-1}			Time (h)	300 ng mL^{-1}			Time (h)	700 ng mL^{-1}		
		OFL	NOR	CPF		OFL	NOR	CPF		OFL	NOR	CPF
AS	0	0	0	0	0	0	0	0	0	0	0	0
	0.02	0.18	0.27	0.25	0.1	0.38	0.62	0.76	0.1	0.99	1.62	1.64
	0.75	0.2	0.31	0.3	0.75	0.46	0.75	0.91	0.75	1.05	1.79	1.85
	1.5	0.22	0.37	0.35	1.5	0.47	0.82	0.97	1.5	1.1	1.94	2.01
	2.25	0.25	0.39	0.46	2.25	0.49	0.84	1.01	2.25	1.27	2.11	2.18
	3	0.27	0.44	0.5	3	0.57	0.97	1.04	3	1.42	2.13	2.23
	24	0.44	0.49	0.59	24	0.77	1.14	1.19	24	1.49	2.23	2.33
	48	0.45	0.5	0.59	48	0.77	1.14	1.19	48	1.5	2.24	2.39
	% biosorbed											
	0	0	0	0	0	0	0	0	0	0	0	0
	0.02	18	25	27	0.1	12	21	24	0.1	14	22	23
	0.75	20	30	31	0.75	16	29	30	0.75	15	26	27
	1.5	22	35	37	1.5	17	30	30	1.5	16	29	29
	2.25	25	39	46	2.25	19	34	34	2.25	18	31	31
	3	27	44	50	3	19	34	34	3	18	32	33
	24	44	49	59	24	25	40	45	24	21	39	42
	48	45	50	59	48	26	40	46	48	21	39	43
	q (mg g_{TSS}^{-1})											
	Time (h)	100 ng mL^{-1}			Time (h)	300 ng mL^{-1}			Time (h)	700 ng mL^{-1}		
		OFL	NOR	CPF		OFL	NOR	CPF		OFL	NOR	CPF
AGS	0	0	0	0	0	0	0	0	0	0	0	0
	0.02	0.08	0.13	0.13	0.1	0.22	0.23	0.45	0.1	0.5	0.49	0.54
	0.75	0.09	0.14	0.15	0.75	0.37	0.37	0.46	0.75	0.51	0.75	0.68
	1.5	0.11	0.16	0.17	1.5	0.47	0.46	0.51	1.5	0.55	0.83	0.74
	2.25	0.11	0.16	0.18	2.25	0.48	0.48	0.52	2.25	0.56	0.84	0.79
	3	0.12	0.18	0.20	3	0.49	0.51	0.55	3	0.77	1.86	1.88
	24	0.15	0.20	0.22	24	0.69	0.82	0.91	24	1.84	2.63	2.87
	48	0.16	0.20	0.23	48	0.69	0.83	0.92	48	1.85	2.73	2.94
	% biosorbed											
	0	0	0	0	0	0	0	0	0	0	0	0
	0.02	2	3	3	0.1	7	8	11	0.1	7	8	15
	0.75	4	4	5	0.75	10	12	15	0.75	12	12	15
	1.5	5	6	7	1.5	13	15	17	1.5	16	15	17
	2.25	6	6	7	2.25	15	16	17	2.25	16	16	17
	3	7	8	9	3	16	17	18	3	16	20	22
	24	9	10	12	24	18	25	28	24	20	27	29
	48	16	20	23	48	19	25	28	48	20	27	30

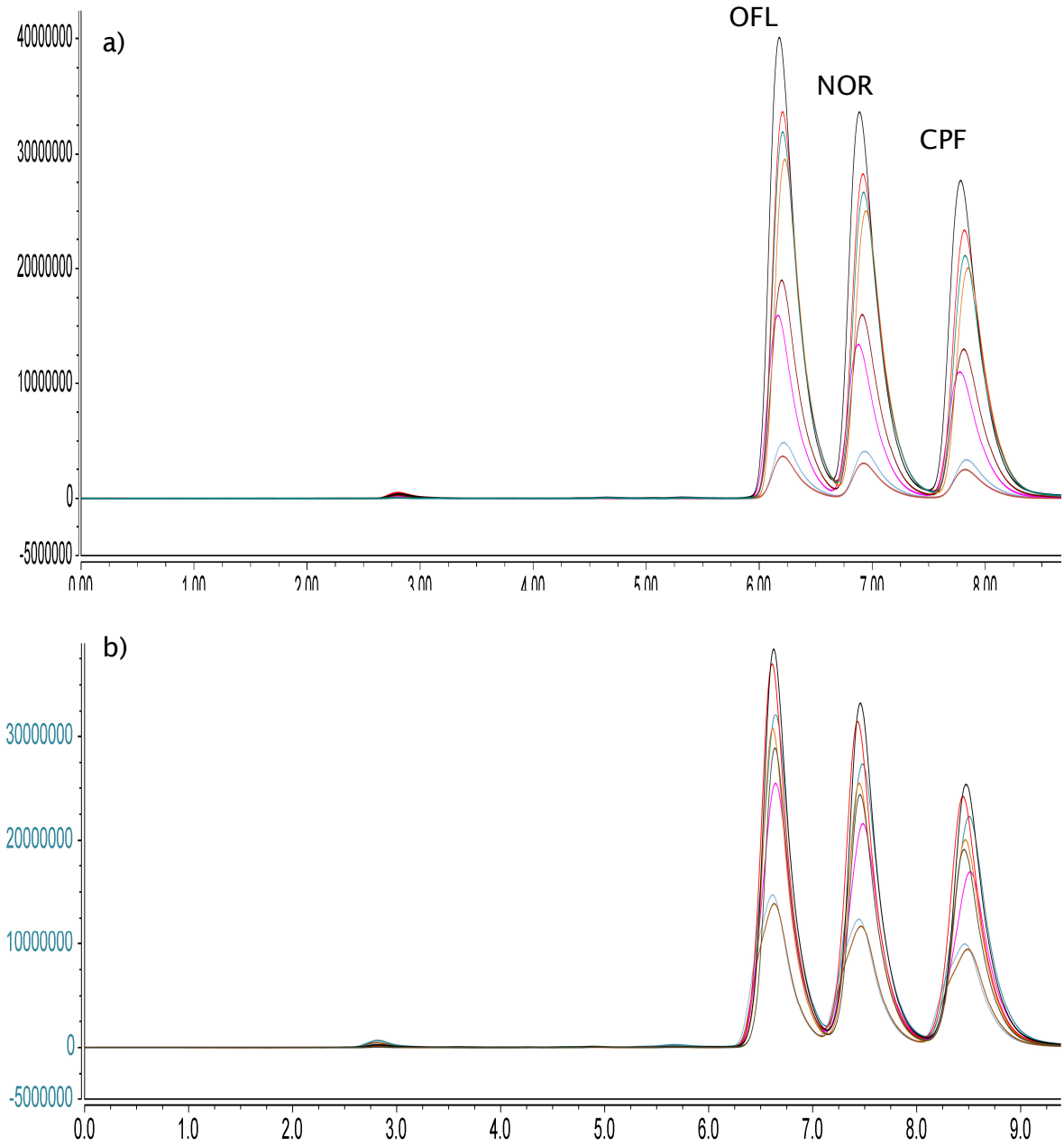


Figure 18. Chromatograms of the free concentration of OFL, NOR and CPF over 48h in 0.01g of biosorbent with Ci = 100 ng mL^{-1} of each FQ : a) AS) and b) AGS (time 0h (black line); time 0.02h (red line); time 0.75h (blue line); time 1.5h (orange line); time 2.25h (brown line); time 3h (pink line); time 24h (light blue); time 48h (light brown)).

Analyzing Table 8 and the chromatograms of Figure 18 it was shown that the profile of biosorption of the three FQs studied in AS and AGS was similar, since when the mass ratio compound / biosorbent increased, q*t* also increased.

Whether using AS either AGS as biosorbent it is noted that during the first 3 h of contact, the biosorption process was faster. After this time has elapsed, the amount of adsorbed compound gradually increases until equilibrium was reached at 24 h. Between 24 and 48 h the biosorption was almost nil, not exceeding 1%. However, to ensure equilibrium between the two phases (liquid-solid) contact time of 48 h was established, since it was necessary to ensure that the duration of the test is sufficient to stabilize the system, regardless of the initial concentration of the FQs.

In the assays with AS (Table 8) it can be seen that changing the initial concentration from 100 ng mL^{-1} to 700 ng mL^{-1} led to an increase in the amount of biosorbed substance after 24h, 0.44 mg g_{TSS}^{-1}, 0.49 mg g_{TSS}^{-1} and 0.59 mg g_{TSS}^{-1} to 1.49 mg g_{TSS}^{-1}, 2.23 mg g_{TSS}^{-1} and 2.33 mg g_{TSS}^{-1} for OFL, NOR and CPF, respectively. This fact can be explained by the increase of the driving force, represented by the increase of the initial concentration of substance analyze, which overcame the resistance to mass transfer of FQs between the liquid medium and the surface of the biosorbent particles (73). However, it is known that at high concentrations (keeping constant the mass of biosorbent), there are fewer active sites available for biosorption occur, and therefore the removal rate at 48 h decreases by increasing the initial concentration of the substance analyze of about 45%, 50% and 49% for OFL, NOR and CPF with Ci =100 ng mL^{-1}, for 21%, 39% and 43%, respectively with Ci = 700 ng mL^{-1}.

In assays with AGS (Table 8) the increase of the initial concentration of each FQs led to the consequent increase of the amount adsorbed but also increased the % of removal at 48h. Thus, for the studied concentrations it was found that higher initial concentration of the tested compounds lead to higher removal. However either for AS or AGS, the removal was not complete even at the lowest concentration (100 ng mL^{-1}).

Analyzing Table 8, it is possible to verify that during the first minute of contact, higher amounts of each compound were biosorbed by AS, e.g. for an initial concentration of 100 ng mL^{-1}, about 18%, 25% and 27% of the initial concentration of OFL, NOR and CPF, was immediately biosorbed. For all other initial concentration studied the profile was similar. For AGS the amount of each compound adsorbed in the first minute was of 10 times lower than for AS in the same conditions. Similar results have been described in the literature when evaluating ammonium adsorption processes in AS and AGS (74). The study of Bassin (2011) also found a higher biosorption in the first 5 min. for AS, while the biosorption by AGS was almost nil. Probably the process of mass transfer would be more difficult in AGS due to its compact structure, delaying the biosorption process.

The chromatograms of Figure 18 also showed of the absence of degradation products, and therefore there was no degradation, for both biosorbents during 48h assay.

The determination of the maximum biosorption per gram of biosorbent was performed varying the initial concentration of each FQ between 100 ng mL^{-1} and 3500 ng mL^{-1}, while keeping all other parameters constant (Fig. 19).

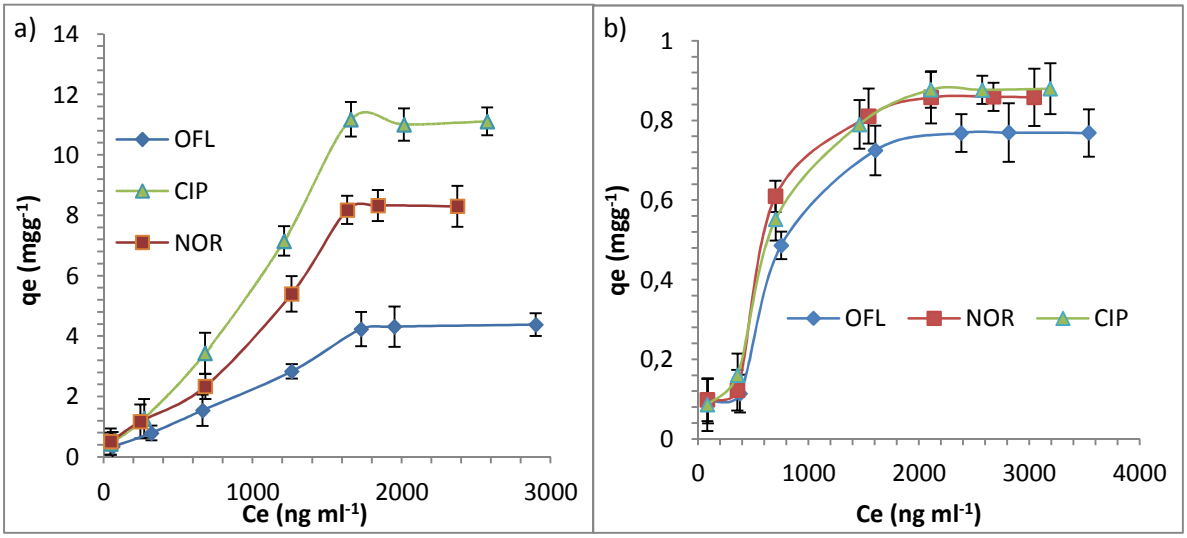


Figure 19. Maximum biosorption of each compound per unit biosorbent, a) AS and b) AGS (24h contact time, Ci = 100, 350, 1000, 2000, 2500, 3000, 3500 ng mL^{-1}).

For AS the maximum biosorption was 4.3 mg g_{TSS}^{-1}, 8.2 mg g_{TSS}^{-1} and 11.1 mg g_{TSS}^{-1} for OFL, NOR and CPF, respectively. Concerning AGS, the maximum biosorption was 0.77 mg g_{TSS}^{-1}, 0.88 mg g_{TSS}^{-1} e 0.89 mg g_{TSS}^{-1} for *OFL*, NOR and CPF, respectively.

According to the results AS had higher biosorption capacity for each studied FQs than AGS.

As mentioned previously, OFL, NOR and CPF are amphocteric molecules that may be present in solution in four different forms depending on the pH of the medium. From Table 4 it is possible to verify that CPF and NOR, at pH 7, are mostly in its zwitterionic and cationic form. Thus the greater was the number of negative charges on the surface the greater will be the number of possible interactions. In addition, as mentioned in the determination of zeta potential (section 3.3.1.2), at pH 7 both AS and AGS had a negatively charged biosorption surface represented by negative zeta potential values. However AS have more negative charges in biosorption surface (- 25.65 mV) compared to AGS (-16.21 mV) providing greater number of possible interaction with the CPF and NOR.

OFL is the FQ less adsorbed onto the AS and AGS. These results were in accordance to what was previously reported by Amorim et al. (51) during AGS-bioreactor operation with the same fluorinated drugs. OFL at pH 7 is mainly present in its anionic form, with a small percentage in the zwitterionic form. Since only of 11% of OFL was in the zwitterionic form responsible for interaction with the biosorbents, possibly this fact could be responsible for the low levels of biosorption, comparing with the biosorption of CPF and NOR.

In addition OFL, NOR and CPF have a common general structure, with possible sites of interaction with the common biosorbent, with a single difference in the R_1 position group, as we can see in Figure 20.

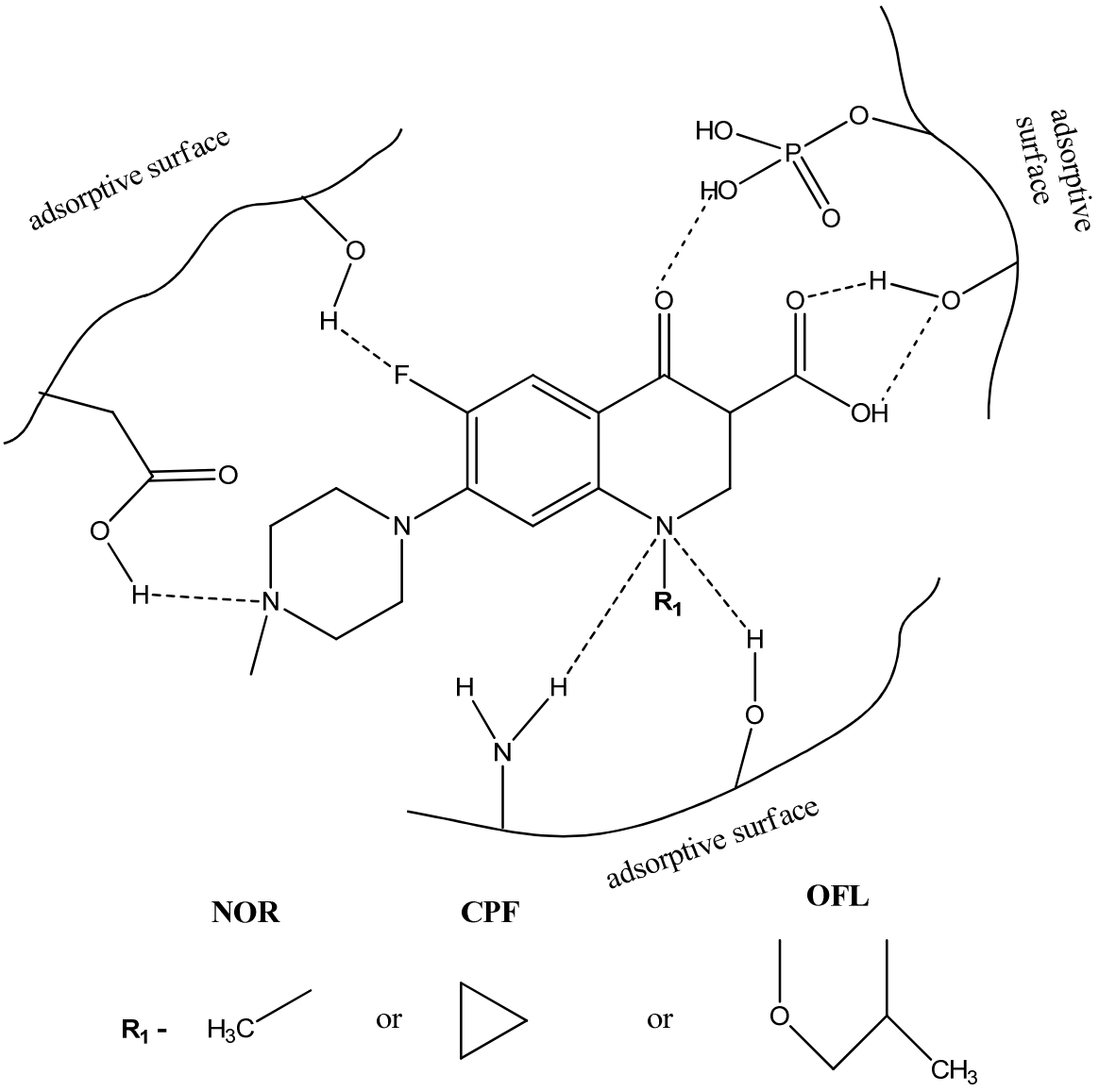


Figure 20. General structure of NOR, CPF and OFL and possible interactions with the surface biosorption.

For OFL, position R_1 is constituted by a isobutoxy bulky group which causes the torsion of the ring, changing the planar structure of the molecule and thereby promoting a steric hindrance of possible hydrogen bonds. At position R_1, NOR and CPF had a cyclopropyl and an ethyl group, respectively that do not alter the planar structure of the molecule. Moreover, the referred substituents are small and therefore did not promote steric hindrance.

The higher biosorption capacity of AS could also be due to the higher concentration of EPS (83 mg g_{TSS}^{-1}) than AGS (68 mg g_{TSS}^{-1}), as mentioned in topic 3.3.1.1 in the biosorbents the EPS is related with the functional ionizable groups which represent possible places available for biosorption. Thus the higher concentrations of EPS, could probably lead to more active sites available for biosorption. The significant concentration of proteins in the surface of AGS (47 mg g_{TSS}^{-1}) also has an effect in the biosorption of OFL, NOR and CPF, since it increases the hydrophobicity of the surface, decreasing negative charges necessary to physically interact with the FQS.

3.3.4 Modulation of the biosorption kinetics

The study of the biosorption kinetics is intended to be an useful tool for the determination of the removal rate of solutes and hence the residence time of the biosorbate in the liquid / solid interface.

The biosorption kinetics of OFL, NOR and CPF in the AS and AGS were analyzed using two models described in the literature. The models used to analyze were the pseudo -first order represented by Equation 2 previously described in the introduction section and the pseudo-second order represented by Equation 3. Both models were fitted to the experimental data using linear analysis.

Table 9 provides a comparison of the biosorption kinetics for both kinetic models chosen for the two biosorbents studied. The pseudo-first order model does not adequately describe the behavior in the whole range of contact time studied being the pseudo-second order model (Eq. 3) the one that better predicts the biosorption behavior since high coefficients of determination were obtained for this last model. The kinetics of pseudo-second order presupposes that the dominant step is the chemical biosorption (56). Chemical biosorption involves electronic transfer, equivalent to the formation of chemical bonds between biosorbate and biosorbent (52).

Table 9. Kinetic parameters of pseudo-first and pseudo-second order models biosorption of OFL, NOR and CPF in AS or AGS.

			Pseudo-first order				Pseudo-second order			
		Ci (ng mL^{-1})	$q_{e,\ exp}$ (mg g_{TSS}^{-1})	$q_{e,\ calc}$ (mg g_{TSS}^{-1})	k_1 (min^{-1})	r^2	$q_{e,\ calc}$ (mg g_{TSS}^{-1})	k_2 (g $mg^{-1}min^{-1}$)	H (mg $g^{-1}min^{-1}$)	r^2
AS	OFL	100	6.4	3.64	0.33	0.973	6.39	28.64	4.02	0.999
		300	0.74	0.59	2.05	0.959	0.78	2.45	3.91	0.999
		700	1.49	1.38	1.31	0.965	2.41	1.47	3.59	0.999
	NOR	100	5.79	3.79	0.85	0.966	5.78	14.06	13.12	1
		300	1.18	0.43	2.54	0.938	1.19	3.94	11.19	0.999
		700	2.01	1.27	1.25	0.989	1.56	1.83	10.47	0.999
	CPF	100	6.07	3. 18	1.75	0.824	6.07	35.02	11.81	1
		300	1.11	0.79	2.57	0.868	1.14	1.94	10.41	0.999
		700	2.03	1.47	1.01	0.858	2.31	1.81	10.07	0.999
AGS	OFL	100	0.31	2.89	1.56	0.545	0.28	1.07	1.08	0.979
		300	0.12	14.12	0.97	0.876	0.12	0.95	0.94	0.999
		700	0.64	2.43	0.64	0.842	0.68	0.31	0.59	0,985
	NOR	100	0.35	2.77	0.91	0.532	0.36	1.55	1.08	0.995
		300	0.14	8.27	0.29	0.894	0.15	1.04	1.07	0.991
		700	0.72	2.41	1.04	0.917	0.72	0.96	1.01	0.993
	CPF	100	0.25	2.81	1.66	0.539	0.28	1.71	0.81	0.977
		300	0.18	5.97	0.19	0.938	0.21	1.03	0.75	0.979
		700	0.71	3.01	0.28	0.931	0.76	0.95	0.64	0.985

k_1: rate constant of pseudo-first order

k_2: rate constant of pseudo-second order

h: Initial biosorption speed

In this study it was found that the rate constants (k_2) of the pseudo-second order kinetic, of each FQs for the two biosorbents, decreases with increasing initial concentrations of the analyze substance from 100 to 700 ng mL^{-1}. The values of the initial biosorption speed ($h = k_2 qe^2$) increased with increasing concentrations of OFL, NOR and CPF. This may be related to the increased in the driving force of biosorption caused by the increase of the initial concentration of the analyze substance which allows molecules to reach the surface

of the biosorbent faster. These results demonstrate that the process dependents on the initial concentration of analyze substance.

However for AS, the obtained values of biosorption constant k_2 were higher than for AGS which indicates a higher biosorption capacity. These results are consistent with what has been reported above.

3.3.5 Study of biosorption equilibrium

The distribution of the solute at the equilibrium is represented by a relationship between the amount of solute in the solid phase (qe) and the solute concentration in the liquid phase at equilibrium (Ce), at constant temperature. This relationship is called isothermal. Although there are several mathematical models capable of representing this equilibrium relationship, only three were studied in this work: the linear isotherm (Eq. 4), the Langmuir isotherm (Eq. 5) and the Freundlich isotherm (Eq. 6), all previously described in the introduction section. The parameters calculated for all the biosorption isotherms are compiled in Table 10.

Table 10. Parameters for linear, Langmuir and Freundlich isotherms (120 rpm, time = 24h).

		Linear		Langmuir			Freundlich		
Biosorbent	FQS	K_d (L g^{-1})	r^2	q_{max} (mg g_{TSS}^{-1})	K_L (L mg^{-1})	r^2	K_f (L g^{-1})$^{(1/n)}$	n	r^2
AS	OFL	0.050	0.948	19.42	0.034	0.998	1.38	0.31	0.842
	NOR	0.131	0.991	24.39	0.371	0.994	1.51	0.81	0.728
	CPF	0.080	0.971	23.87	0.020	0.998	1.35	0.73	0.852
AGS	OFL	0.007	0.979	4.59	0.004	0.995	10.47	1.31	1.000
	NOR	0.007	0.964	3.17	0.003	0.956	6.61	1.64	0.996
	CPF	0.006	0.933	2.34	0.002	0.978	4.65	2.01	0.995

K_d: Distribution coefficient; **n**: Freundlich constant;
K_l: Langmuir constant **K_f** : Freundlich biosorption coefficient
q_{max}: capacity biosorption monolayer

For AS, the Langmuir model leads to higher values of r^2, and was therefore the model that best predicts the behavior of the experimental points. This model assumes that the biosorption energy of each molecule is equal and independent of the degree of surface coverage. Assumes also that the biosorption occurs at specific sites of the biosorbent and

that, once busy, are not available to biosorb any more molecule (each site has the ability to biosorb only one molecule at a time). The model also assumes that there is no interaction between adsorbed molecules and neighboring sites. In sum, this model assumes that the surface biosorption is homogeneous and monolayer biosorption occurs with the occupation of all active sites which lead to saturation and thus a limit of biosorption should be achieved (52, 55). This isotherm can be applied to the results of biosorption for AS in the first minute of contact. The active sites have equal affinity and were rapidly occupied in the first minutes of contact. When these sites were occupied biosorption no longer occurred, reaching saturation.
For active AGS the Freundlich model was the one that best fits with the experimental points and showed better r^2. Furthermore values of n (degree of biosorption) higher than 1 made this isothermal favorable. This model is applied to describe systems where biosorption occurs on heterogeneous surfaces with biosorption sites with different levels of interaction.

Therefore, this model assumes that the locations where the interaction is strongest are occupied first, and that the interaction strength decreases with increasing degree of occupation of the active sites (52). Furthermore it adequately describe the reversible biosorption and is not restricted to the formation of monolayers (55). This fact may justify the isothermal biosorption profile of AGS. The biosorption of FQs was more pronounced during the first 3h of contact due to the occupation of biosorption sites with higher affinity and lower affinity sites were probably occupied more slowly, thus having a slow biosorption from that time onwards.

3.3.6 Desorption assays

Desorption assays with AGS were performed by altering the pH of the medium to promote desorption of each FQs. The quantities adsorbed/desorbed at different pH, for the same initial concentration of each FQ (300 ng mL^{-1}) are shown in Figure 21.

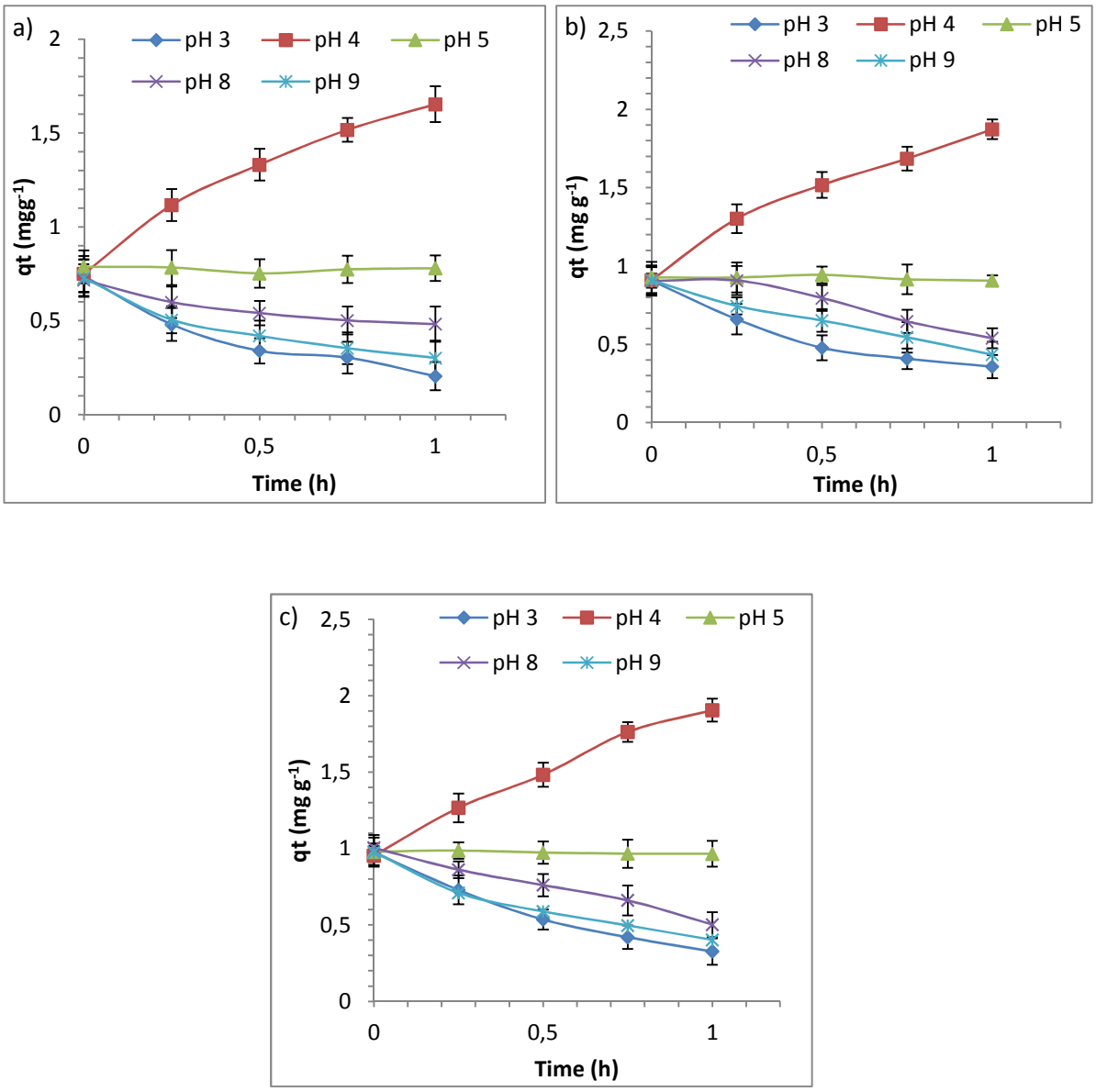


Figure 21. pH effect to a) OFL, b) NOR and c) CPF (1h biosorption Ci = 300 ng mL^{-1}).

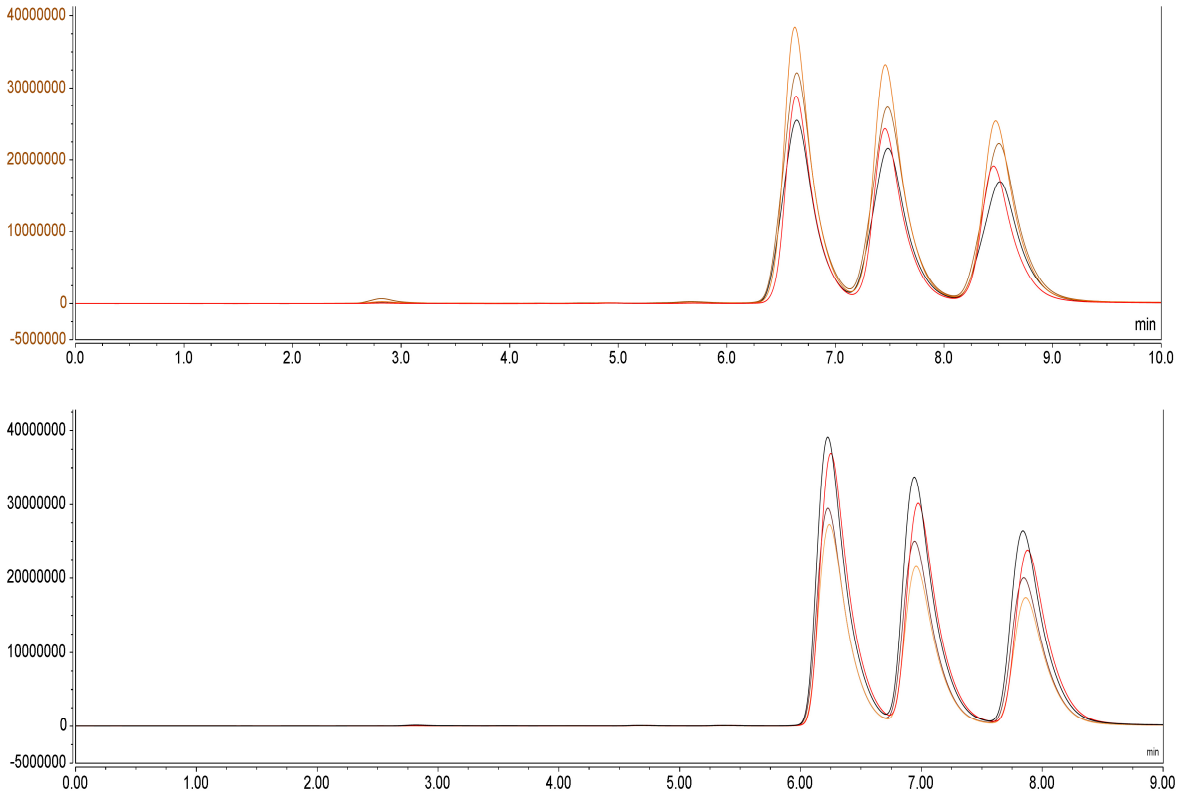


Figure 22. Chromatograms of the free concentration of OFL, NOR and CPF in 0.01g of AGS, with Ci = 300 ng mL^{-1} of each FQ, at different pH of the medium : a) pH 3; b) pH 4 (time 0h (black line), time 0.25h (red line); time 0.5h (brown line); time 1h (orange line).

On time zero was presented the amount of previously adsorbed FQs (0.78 mg g_{TSS}^{-1} 0.91 mg g_{TSS}^{-1} and 0.98 mg g_{TSS}^{-1}) after 48 h at pH 7 with Ci = 300 ng mL^{-1} of OFL, NOR and CPF, respectively. After 48h at pH7 the biosorption was not complete and there is still free FQs in the medium during the experiment at different pH. As shown in Figure 20, adjusting pH to 3, 8 or 9 desorption of OFL, NOR and CPF was promoted. The highest degree of desorption after 1h occurred at pH 3 (53% OFL, 60% NOR and 63% CPF) followed by pH 9 (45% OFL, 53% NOR and 55% CPF) and pH 8 (41% OFL, 48% NOR and 50% CPF).

OFL, NOR and CPF possess ionizable functional groups, amine and carboxyl groups, which impart confer an amphoteric behavior according to the pH of the medium. As observed in Table 4 OFL, NOR and CPF can be in its cation, anion, neutral and/or zwitterionic form according to the pH of the medium. According to the literature, the mechanism of absorption for all three studied FQs by ion exchange is favored when the compounds are in the form of cation, or when the pH of the medium is acidified (58, 63).

However, the pH of the medium has effect on the OFL, NOR and CPF but also on the biosorbent surface. AGS are also composed of ionizable functional groups and therefore

also had a positive / negative charge in accordance with pH of the medium (74, 75). The charge on the surface of the AGS was determined at various pH and the results of zeta potential were previously presented on the section 3.3.1.2. Given the % of the anionic, cationic and zwitterionic forms of each FQs at different pH and taking into account the charge on the biosorbent surface (zeta potential) it is possible to understand the results obtained in the desorption tests.

At pH 4 biosorption of all tested FQs occurred. After one hour, at pH 4, biosorption was higher than that occurred after 48 h, at pH 7 (OFL = 0.77 mg g_{TSS}^{-1}, NOR = 0.91 mg g_{TSS}^{-1} and CPF = 0.93 mg g_{TSS}^{-1}). This result was probably due to the presence of protonated OFL, NOR and CPF in solution and the significant number of negative charges on the surface of the AGS, represented by a zeta potential of -12.28 mV, so the interaction between the compound and the biosorbent surface was greater than that existing at pH 7 promoting the biosorption. Although at pH 7 the zeta potential was higher (-16.21 mV), the three FQs were mostly in their zwitterionic form with a small % in the cationic forms. As such the interaction between the AGS surface and the FQs was lower compared to that registered at pH 4.

At pH 5 there were no significant changes because the values of negative charge on the biosorbent surface (-15.14 mV) were similar to those determined at pH 7 (-16.21 mV) wich did not significantly increase the number of places available to adsorb the fluorinated drugs.

At pH 8 and 9 desorption occurred probably because of the excess of negative charges on the biosorbent surface (-17.93 and -19.01 mV) that repel FQs that were mainly in their anionic form promoting their desorption.

At pH 3 higher desorption was achieved, this was because at the extremes pH there is a destruction of the granular structure and hence the biosorptive surface.

The chromatograms (Fig. 22) also shows that there is no detection of degradation products, at different pH of the medium, in other words, there is no degradation, during 1h assay.

4. Conclusions

4. Conclusions

This multidisciplinary work involved analytical chemistry and microbiology techniques in order to better understand biosortion of fluoroquinolonas. A HPLC-FD method was developed and validated according to the International Q2B ICH (1996) and FDA (2001) guidelines for detecting and quantifying OFL, NOR and CPF. The chromatographic method showed to be efficient for monitoring all the target compounds in all the performed biosortion tests. The adsorption capacities of AS and AGS to remove OFL, NOR and CPF were evaluated. The biosortion process was found to be dependent on the concentration of the biossorbents and the concentration of FQs. The initial concentrations used in the tests of biosortion aimed to mimic the environmental conditions and were in the order of ng mL^{-1}.

AS showed better performance for the removal of OFL, NOR and CPF relative to AGS. However, the complete removal was not achieved, in the studied concentrations. We purpose that the higher biosortion capacity of AS is mainly due to the negative charge of the biosortion surface, represented by a zeta potential of -25.65 mV, at pH 7. OFL is the less biosorbed FQ, both onto the AS and AGS, because OFL at pH 7 is mainly present in its anionic form, with a small percentage in the zwitterionic form.

Studies of biosortion kinetics in AS and AGS indicated the kinetic equation of pseudo-second order as the most appropriate to settle the experimental points. The equilibrium data for AS showed a better fit to the Langmuir model, while the AGS model that presented better fit, for all experimental points, was the Freundlich model. The influence of pH on the process of biosorption was also studied. We found that in the alkaline medium (pH 8 and 9), OFL, NOR and CPF tend to desorb from AGS and in the acidic medium (pH 4) promoted the adsorption of OFL, NOR and CPF. However at pH ≤ 3 high desorption was observed due to the destruction of the granular structure and hence the biosorptive surface.

Further studies on the mechanisms of biosortion and a detailed characterization of the biosorbent at physical, chemical and microbiological level need to be carried out. It is also important to evaluate the reuse of the biosorbents after their regeneration by pH change and to explore other possible regeneration processes.

5. References

5. References

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